ELSEVIER

Carbohydrate Polymers 46 (2001) 323–330

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Synthesis and bioactivities of poly(ethylene glycol)–chitosan hybrids

T. Muslim[a], M. Morimoto[a], H. Saimoto[a], Y. Okamoto[b], S. Minami[b], Y. Shigemasa[a,*]

[a]*Department of Materials Science, Faculty of Engineering, Tottori University, Koyama, Tottori 680-8552, Japan*
[b]*Department of Veterinary Surgery, Faculty of Agriculture, Tottori University, Koyama, Tottori 680-8553, Japan*

Accepted 25 October 2000

Abstract

Poly(ethylene glycol)–chitosan hybrids of various molecular weights having different degree of substitution were synthesized, by reductive *N*-alkylation of chitosan with poly(ethylene glycol) aldehyde, to study their bioactivities. The influence of these chitosan derivatives on the reactive oxygen species generation from canine polymorphonuclear leukocyte cells was investigated in vitro by chemiluminescence response. Reactive oxygen species generation by the influence of poly(ethylene glycol)–chitosan hybrids was decreased with the increase of degree of substitution. The reduction of interaction of poly(ethylene glycol)–chitosan hybrids with polymorphonuclear leukocyte cells might be caused by the decrease of amino group in chitosan main chain and increase of the steric hindrance by poly(ethylene glycol) chain. The influence of the poly(ethylene glycol)–chitosan hybrids on complement component C3 activation was investigated by single radial immunodiffusion method. Influence on complement component C3 activation by poly(ethylene glycol)–chitosan hybrids was almost same as chitosan.

Keywords: Chitosan; Poly(ethylene glycol); Bioactivity; Chemiluminescence; Single radial immunodiffusion method; Polymorphonuclear leukocyte; Complement

1. Introduction

Due to their biodegradability, biocompatibility and bioactivities, chitin and chitosan have created significant interest in biomedical applications (Shigemasa & Minami, 1995). Biomaterials of chitin and chitosan have been used in veterinary practice to activate host defenses in preventing infection and to accelerate the wound healing (Shigemasa & Minami, 1995). Chitin and chitosan act as stimulants for polymorphonuclear leukocytes (PMN) and macrophages, inducing the migration of PMN cells into the wounds and then inducing active biodebridement by these cells. Canine PMN cells can interact with chitosan particles by opsonization with complements in serum. This interaction accelerates the phagocytic activity of PMN cells (Minami et al., 1993). During phagocytosis process, bactericidal toxic reactive oxygen species ($OH^{\cdot}$, O_2^1, O_2^-, H_2O_2) are generated, which sterilize the wound portion (Allen, Stjernholm & Steele, 1972; Babior, 1984).

To get chitin or chitosan derivatives having improved hydrophilic character, some approaches had been made for grafting hydrophilic polymers onto chitin and chitosan (Aoi, Takasu & Okada, 1994; Blair, Guthrie, Law & Turkington, 1987; Hoffman et al., 1997; Kurita, Yoshida & Koyama, 1988; Yalpani, Marchessault, Morin & Monasterios, 1991). Poly(ethylene glycol) (PEG) is a polymer widely used as pharmacological product showing hydrophilicity and biocompatibility with low biodegradability. Harris et al. (1984) published on the synthesis of PEG–chitosan derivative by the modification of chitosan with reductive alkylation of amino group by PEG–aldehyde. Aiba (1993) reported on the synthesis of PEG–chitosan derivatives by reacting cyanuryl trichloride activated monomethoxypoly(ethylene glycol) (mPEG-C) and monomethoxypoly(ethylene glycol)-succinimidyl succinate (mPEG-S) with amino group of chitosan. Saito, Harris and Hoffman (1997) synthesized graft copolymer of PEG on a chitosan backbone (PEG-*g*-chitosan) with methoxy-PEG-*p*-nitrophenyl carbonate as a possible material for delivery of anionic drugs. Sugimoto, Morimoto, Sashiwa, Saimoto and Shigemasa (1998) prepared and characterized different types of water-soluble chitin/chitosan–PEG hybrids and studied their solubility. Sugimoto and Shigemasa (1998) also studied the interaction between chitin/chitosan–PEG hybrids and acrylic emulsion. Aggregation of PEG-grafted chitosan in aqueous solution was studied for using it as drug carrier (Ouchi, Nishizawa & Ohya, 1998). Amiji (1997) reported on the synthesis of anionic poly(ethylene glycol) derivative for the modification of the chitosan surface in

* Corresponding author. Tel.: +81-857-31-5254; fax: +81-857-31-5254.
E-mail address: shigemas@chem.tottori-u.ac.jp (Y. Shigemasa).

0144-8617/01/$ - see front matter
PII: S0144-8617(00)00331-3

Table 1
Preparation of PEG-aldehyde by DMSO/acetic anhydride oxidation

Run no.	PEG[a] (M_n)	Molar ratio of Ac_2O/–OH of PEG[b]	Temperature (°C)	Time (h)	DC[c]	Yield[d] of PEG-aldehyde (%)
1-1	2000	21.1	18	9	0.40	25
1-2	2000	10.5	20	9	0.33	28
1-3	550	4.9	25	9	0.44	43
1-4	550	5.2	50	3	0.92	92

[a] PEG means poly(ethylene glycol) monomethyl ether.
[b] Dry DMSO and dry $CHCl_3$ were used as solvent.
[c] DC means the degree of conversion from –OH to –CHO, and –CHO was estimated by Schales' method.
[d] Yield is indicated with the amount of PEG-aldehyde produced from PEG, calculated by DC.

blood-contacting applications. Dal Pozzo, Vanini, Fagnoni, Guerrini, De Benedittis and Muzzarelli (2000) prepared and characterized the poly(ethylene glycol)-crossed reacetylated chitosans to study their solubility and biodegradability and biocompatibility.

Various reports have been published related to the bioactivities of different chitosan derivatives (Dutkiewicz, Szosland, Kucharska, Judkiewicz & Ciszewski, 1990; Murata, Saiki, Nishimura, Nishi, Tokura & Azuma, 1989; Nishimura, Nishi, Tokura, Nishimura & Azuma, 1986; Tanigawa, Tanaka, Sashiwa, Suzuki, Saimoto & Shigemasa, 1992; Terada, Morimoto, Saimoto, Okamoto, Minami & Shigemasa, 1999; Yalpani, Johnson & Robinson, 1992). Very little information has been found related to the bioactivity of PEG–chitosan hybrid (Li et al., 1999) and therefore in the present study, we have focused on the bioactivities of PEG–chitosan hybrids.

2. Experimental

2.1. Materials

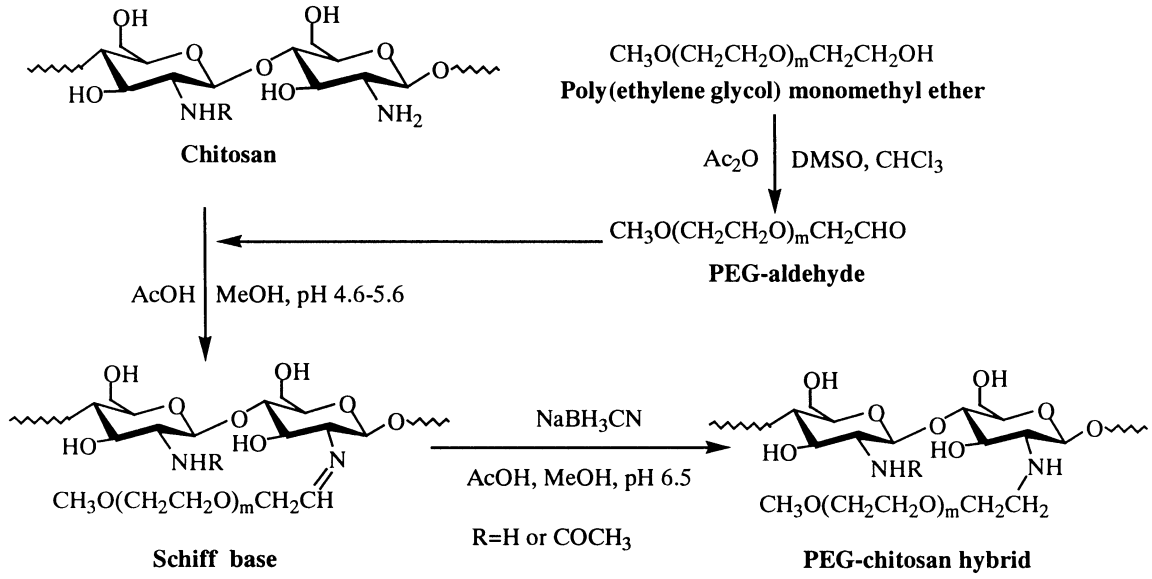
Chitosan Flonac C was procured from Kyowa Technos Co., Japan. The molecular weight M_n was 25,000 and M_w was 91,000 (determined by GPC with pullulan as standard) and the degree of deacetylation (DDA) was 86% (determined by ^1H NMR). Poly(ethylene glycol) monomethyl ethers of M_n 550 and 2000 were from Aldrich Chemical Co., USA. Agar powder and zymosan A were purchased from Sigma Chemical Co., USA. Goat antiserum to dog complement component C3 (total protein = 44.44 mg/ml) was purchased from ICN Pharmaceuticals, Inc., USA. Powder Hank's solution and HEPES (*N*-2-hydroxyethylpiperine-*N'*-2-ethanesulfonic acid) were purchased from Nissui Pharmaceutical, Co., Ltd, Japan and Wako Chemical Co., Japan, respectively. Luminol and sodium azide were obtained from Nacalai Tesque, Japan. All the other reagents and chemicals were of analytical grade or higher.

2.2. Preparation of PEG-aldehyde

Poly(ethylene glycol) monomethyl ether having M_n 2000 and 550 were oxidized to PEG-aldehyde with acetic anhydride and DMSO according to the method described by Sugimoto (Harris et al., 1984; Sugimoto et al., 1998). The reaction conditions are shown in Table 1. The final product was the mixture of PEG and PEG-aldehyde. The degree of the conversion (DC) from hydroxyl group to aldehyde group was estimated by Schales' method with the calibration curve of glutaraldehyde (Imoto & Yanagishita, 1971). Yield was calculated in terms of amount of produced PEG-aldehyde. The DC and yields are summarized in Table 1. The low temperature or room temperature was preferred for the oxidation reaction since there might be a chance of excessive reaction. Moisture free reaction condition was maintained. In case of PEG having M_n 2000, after purification by the reprecipitation method, a white solid was obtained as final product. PEG of M_n 550 was liquid and produced liquid PEG-aldehyde. It could not be separated from reaction mixture and was used without further purification.

2.3. Preparation of PEG–chitosan hybrid

The preparation of PEG–chitosan hybrids was performed by the method described by Sugimoto (Harris et al., 1984; Sugimoto et al., 1998). PEG–chitosan hybrids of different degree of substitution (DS) value and different molecular weight of PEG chain were prepared. The preparation of PEG–chitosan hybrid is shortly outlined in Scheme 1. The reaction conditions are shown in Table 2. Sugimoto studied the preparation method extensively along with solubility of the products. He showed that most of the PEG–chitosan hybrids having long PEG side chain (such as M_n 2000 and 5000) were water-soluble and where as PEG–chitosan hybrids having short PEG side chain (such as M_n 550) were mostly water-insoluble. Of course, DS was the main determining factor for solubility. From that experience, preparation of two types of PEG–chitosan hybrids, water-soluble and water-insoluble, was planned. PEG-aldehyde of M_n 2000 was used for the preparation of water-soluble PEG–chitosan hybrids having various DS. Similarly, PEG-aldehyde of M_n 550 was used to prepare water-insoluble PEG–chitosan hybrids having different DS. All PEG–chitosan hybrids were characterized



Scheme 1.

by ^{1}H NMR. In all cases, the characteristic peak for –NH–CH_2CH$_2$O–, methylene proton of PEG and for chitosan H-1 were identified and DS was calculated from ^{1}H NMR's peak intensities by Sugimoto's method (Sugimoto et al., 1998). Yield was calculated from the amount-recovered chitosan in PEG–chitosan hybrids by using DS value. All the DS and yields are presented in Table 2. In many cases, a minor fraction as water-soluble or water-insoluble fraction was obtained at the purification step. The minor fractions were ignored in our study. It is necessary to note that all PEG–chitosan hybrids were washed extensively several times with acetone and filtered to remove all unreacted PEG remained in the hybrids. The PEG–chitosan hybrids having PEG of M_n 2000 showed their water solubility (1 mg/ml) in DS ranging from 0.06 to 0.74. The PEG–chitosan hybrids having PEG size of M_n 550 showed their water insolubility (1 mg/ml) in DS ranging from 0.05 to 0.31.

Table 2
Preparation of PEG–chitosan hybrid[a]

Run no.	PEG-aldehyde (M_n)	Molar ratio		PEG–chitosan hybrid		
		–CHO of PEG/–NH_2 of chitosan	$NaCNBH_3$/–CHO of PEG	DS[b]	Yield (%)[c]	Weight ratio PEG/hybrid[d]
2-1	550	0.82	9.7	0.05	70	0.14
2-2	550	0.61	10.2	0.08	68	0.21
2-3	550	0.53	19.8	0.11	59	0.27
2-4	550	0.88	7.8	0.17	37	0.64
2-5	550	0.77	9.7	0.25	64	0.55
2-6	550	0.78	8.7	0.31	35	0.49
2-7	2000	0.21	7.4	0.06	92	0.58
2-8	2000	0.13	7.1	0.10	51	0.55
2-9	2000	0.28	6.5	0.19	56	0.70
2-10	2000	0.30	6.0	0.28	49	0.77
2-11	2000	0.40	6.1	0.32	72	0.79
2-12	2000	0.50	6.2	0.42	48	0.83
2-13	2000	0.60	6.3	0.49	51	0.85
2-13	2000	0.70	6.4	0.57	39	0.87
2-15	2000	0.80	6.5	0.74	35	0.90

[a] Chitosan: Flonac C; $M_n = 25,000$; $M_w = 91,000$; DDA = 86% (determined by ^{1}H NMR). Reaction condition: solvent, 2:1 mixture of 2% acetic acid and methanol; pH = 6.5; room temperature; reaction time = 18 h.

[b] DS means the degree of substitution of PEG to monosaccharide residue of chitosan determined by ^{1}H NMR.

[c] Yield is indicated with the amount of recovered chitosan in PEG–chitosan hybrid calculated by DS.

[d] The weight ratio means the weight ratio of PEG in PEG–chitosan hybrid calculated by DS and M_n of PEG.

2.4. ^1H NMR spectroscopy

^{1}H NMR spectra were recorded on a JEOL JNM-GX270 spectrometer following the method described by Sugimoto (Sugimoto et al., 1998). Samples were dissolved in D_2O containing a few drops of 20 wt% DCl/D_2O or in 20% DCl/D_2O (Shigemasa, Mastuura, Sashiwa & Saimoto, 1996). The chemical shifts were referenced from DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). ^{1}H NMR data of PEG–chitosan hybrid (D_2O containing a few drops of 20 wt% DCl/D_2O, DSS): δ 4.5–5.1 (br.d., H-1), 3.78–4.21 (H-3, H-4, H-5, H-6, H-6′ and –OCH_2–), 3.36 (–OCH_3), 3.12 (br.s., H-2), 2.65 (br.s., –NH–CH_2–), 2.04 ppm (br.s., –COCH_3). ^{1}H NMR data of PEG–chitosan hybrid (20 wt% DCl/D_2O, DSS): δ 5.5–4.9 (H-1), 3.93–3.72 (H-3, H-4, H-5, H-6, H-6′ and –OCH_2–), 3.40 (–OCH_3), 3.12 (br.s., H-2), 2.94 (br.s., –NH–CH_2–), 2.14 ppm (br.s., –COCH_3).

2.5. Chemiluminescence assay

Effect of the PEG–chitosan hybrids on the reactive oxygen species generation of canine PMN was evaluated by luminol-aided chemiluminescence method (Li et al., 1999; Makimura & Sawaki, 1992).

2.5.1. Reagent

Hanks' balanced salt solution (*HBSS*): powder Hanks solution (9.8 g) was dissolved in 1000 ml of distilled water and HEPES (1.2 g) was added to it. The mixture was stirred at room temperature until dissolution. The pH of the solution was adjusted to 7.3 with 1 M aqueous sodium hydroxide, and then sterilized by filtration with 0.45 μm Millipore filter.

Zymosan suspension: zymosan (100 mg) was suspended in a physiological saline (10 ml). The suspension was heated at 100°C for 60 min and then centrifuged at 2000 rpm for 10 min. The precipitate was suspended in HBSS (10 ml), stored at −20°C, and diluted 10 folds with HBSS before use.

Luminol solution: luminol (100 mg) was added to sterilized distilled water (50 ml) and mixed well. Then triethylamine (50 μl) was added to it. After sufficient shaking and ultrasonication for 45 min at 50°C, the mixture was passed through a 0.45 μm Millipore filter to remove insoluble luminol particles and stored at −20°C.

Canine blood: for each set of experiment, fresh canine blood (5 ml) was used. Blood was collected from the jugular vein of healthy adult dog with heparin treated sterile syringe.

2.5.2. Chemiluminescence measurement

The chemiluminescence (CL) response of the luminol excited by the reactive oxygen species was measured with a Biolumat LB 9505 (Berthold Co., Germany). Zymosan was used as reference material. The CL intensity was defined as a value of peak counts of the CL response at maximum point. The CL intensity for zymosan was defined as 100%. The peak CL count (peak CL cpm/1000 PMNs) and relative CL intensity for the samples were calculated with the following equations:

$$\text{Peak CL count} = \{\text{CL value} \times (1 - \text{baseline index})/G \times V\} \times 1000 \quad (1)$$

where, base line index = CL baseline value/CL value; G is the PMN count per microliter and V is the whole blood volume (100 μl).

$$\text{Relative CL intensity (\%) (\textit{In the absence of zymosan})} = (\text{Peak CL count for sample}/\text{Peak CL count for zymosan}) \times 100 \quad (2)$$

$$\text{Relative CL intensity (\%) (\textit{In the presence of zymosan})} = \{\text{Peak CL count for (sample + zymosan)}/\text{Peak CL count for zymosan}\} \times 100 \quad (3)$$

The methods used in the CL measurement of different samples is described below.

In the absence of zymosan: 100 μl of canine blood was diluted with 400 μl of HBSS. After incubation of the diluted blood for 15 min at 37°C, 20 μl of aqueous luminol (2 mg/ml) was added, and then incubated for 3 min again. The CL measurement was started for 1 min to define the baseline, followed by addition of 50 μl of PEG–chitosan hybrid suspension in HBSS (1 mg/ml). The CL response was recorded for 30 min continuously. The CL response for zymosan was also recorded in the same manner. Relative CL intensity (%) was calculated by using Eq. (2).

In the presence of zymosan: 100 μl of canine blood was diluted with 350 μl of HBSS. To the diluted blood, 50 μl of solution of PEG–chitosan hybrid in HBSS (1 mg/ml) was added. After incubation for 15 min at 37°C, 20 μl of aqueous luminol was added, then incubated for 3 min again. The CL measurement was started for 1 min to define the baseline, followed by addition of zymosan suspension in HBSS (50 μl, 1 mg/ml). The CL response was recorded for 30 min continuously. The CL response for zymosan (without addition of PEG–chitosan hybrid) was also recorded in same manner. Relative CL intensity (%) was calculated by using Eq. (3).

2.6. Single radial immunodiffusion method for complement component C3 activation assay

Complement activation was determined by change plasma complement component C3 concentration using the single radial immunodiffusion method (SRID) (Minami et al., 1998; Vaerman, 1981).

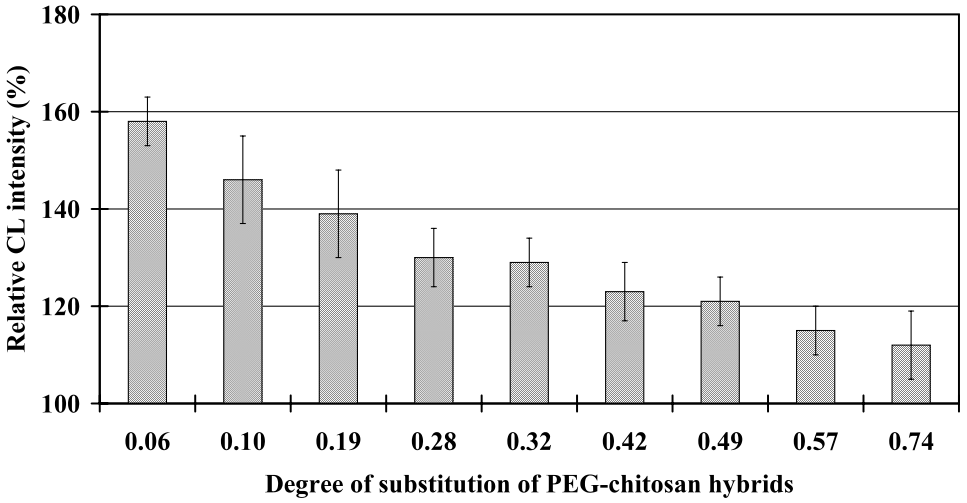


Fig. 1. Effect of water-soluble PEG–chitosan hybrids on canine PMN (whole blood) chemiluminescence (CL) response. CL response for PEG–chitosan hybrids having different degree of substitution was measured in presence of zymosan. The relative CL intensity (%) was calculated with respect to zymosan (100%). Data are displayed as mean ± SD ($n = 8$).

2.6.1. Plasma

Canine blood sample was divided into cooled test tubes stored in the ice bath and then centrifuged at 4°C, 3000 rpm for 15 min. The collected plasma was separated into micro-tubes and was kept in deep freezer at −80°C until use.

2.6.2. Sample

The stored plasma was dissolved in water bath at 37°C. Plasma of 200 μl was taken in a test tube. This test tube was for the measurement of basic value that was original C3 concentration in the plasma. Physiological saline of 400 μl was added to it. This solution was used as a control in SRID. The sampling method for chitosan (Flonac C) and any water-insoluble PEG–chitosan hybrid was followed: 2 or 4 mg of sample was suspended in 400 μl of physiological saline and ultrasonicated for 1 h. Canine plasma of 200 μl was added to it and incubated at 37°C for 40 min. The tube was centrifuged for 1 min and the supernatant was collected for the estimation of C3 concentration by SRID.

2.6.3. SRID method

Gel was made by dissolving 1.5 g of agar in 100 ml of phosphate buffer (pH 7.2, 0.02 M) with the microwave treatment for 2 min. Agar solution of 15 ml was transferred to a beaker (all glassware were preheated at 50°C) and immediately heated on a water bath at 50°C. Then 0.1 ml of sodium azide solution (300 mg/ml), and 375 μl of anti-C3 serum was added to it and mixed well. To make gel plate, the gel was poured into a square glass box (15 cm × 7.5 cm × 0.18 cm, preheated at 50°C) and immediately cooled in an ice-bath. The gel plate was taken off on a flat glass plate, and was cut with a sharp blade into the 1.5 cm × 1.5 cm wide and a 1.5 mm diameter hole was made in the each plate center. The gel kept in a humidified box. Each plasma sample (for control and samples) of 2.5 μl was placed in the center of the hole and the plate was incubated at 4°C for 48 h. After incubation, the size of precipitation ring was measured by microscopic observation.

The calculation formula of C3 value is as follows: Ring area $= \pi(R_a^2 - R_b^2)$, where R_a is a half of ring diameter and R_b is a half of center hole diameter. Each ring area (C3 value) was converted into percentage by each base value of the control ring area (100%).

3. Results and Discussion

3.1. Effect of PEG–chitosan hybrids on the chemiluminescence of canine PMN cells

During the interaction of sample with whole canine blood, PMN cells of the canine blood phagocytized the sample. In this phagocytosis process, reactive oxygen species were generated. When luminol was excited energetically by reaction with reactive oxygen species, returned to ground state, photons were emitted and could be measured as chemiluminescence (Makimura & Sawaki, 1992; Trush, Wilson & Dyke, 1978). Relative CL intensities for water-soluble and water-insoluble PEG–chitosan hybrids were estimated by two different methods.

CL response of water-soluble PEG–chitosan hybrids was measured in the presence of zymosan. CL response for zymosan only was defined as 100% and result is expressed with comparison to this. The result for every sample showed relative intensity over 100% and varied from 158 to 112%. The relationship between the relative CL intensity with DS value is shown in Fig. 1. The reactive oxygen species generation by the influence of PEG–chitosan hybrids was decreased with the increase of DS by PEG onto chitosan,

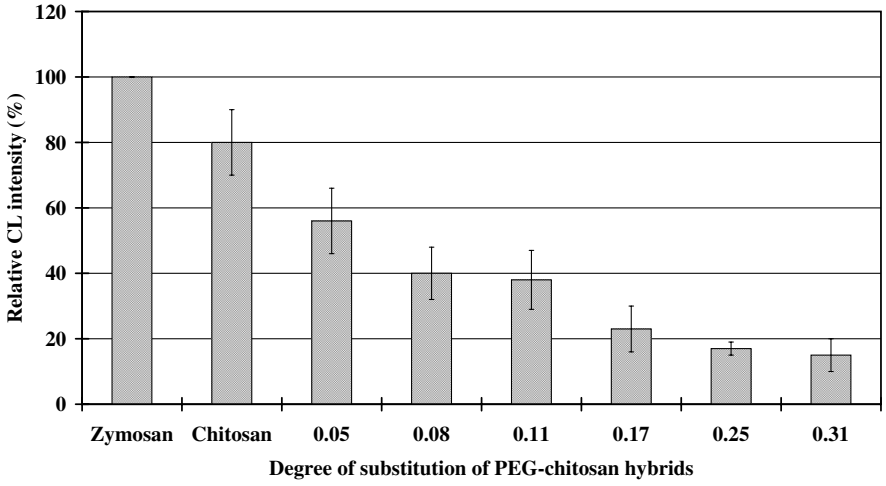


Fig. 2. Effect of water-insoluble PEG–chitosan hybrids and chitosan on canine PMN (whole blood) chemiluminescence (CL) response. CL response for chitosan and water-insoluble PEG–chitosan hybrids having different degree of substitution was measured in absence of zymosan. The relative CL intensity (%) was calculated with respect to zymosan (100%). Data are displayed as mean ± SD ($n = 6$).

and the decrease of amino group amount. Recently, our laboratory reported on the evaluation of bioactivities of water-soluble chitosan–sugar hybrids by measurement of chemiluminescence (CL) response (Li et al., 1999). The report showed that CL response depended on DS and it decreased with increasing DS value. The water-soluble hybrids sensitized the PMN cells membrane and activated phagocytosis of PMN cells for zymosan by a priming effect (Pabst & Johnson, 1980). It was suggested that the number of amino groups of glucosamine residues of the hybrids would be important for the priming effect of water-soluble hybrids. Another report showed that glucosamine oligomer induced enhancement of CL response of canine PMN cells to zymosan by priming effect. But CL response did not change when *N*-acetylglucosamine oligomers were added to PMN cells (Usami, Okamoto, Takayama, Shigemasa & Minami, 1998). Water-soluble glucosamine oligomer and sugar–chitosan hybrids enhanced CL response by priming effect and the presence of amino group was essential for this effect. In the present study, it is evidenced that the amount of amino groups in chitosan main chain of water-soluble PEG–chitosan hybrids might play a significant role on the generation of reactive oxygen species by PMN cells. We also predict that the water-soluble PEG–chitosan hybrids might stimulate the PMN cells by priming effect.

CL response of water-insoluble PEG–chitosan hybrids, was measured in the absence of zymosan. CL response for zymosan was also defined as 100%. CL responses were varied from 56 to 15%. The relationship between the relative CL intensity with DS value is shown in Fig. 2. The CL intensity was markedly decreased with increase of DS value. The reactive oxygen species generation by the influence of PEG–chitosan hybrids was decreased with the decrease of amino group amount, and the increase of PEG amount in the hybrid. In an early report, it was found that the partially deacetylated chitin (DAC) with a high degree of deacetylation (DDA) more effectively inhibited bacterial growth than those with a low DDA, suggesting that the number of amino groups in the DAC was correlated with the extent of bacterial growth (Tanigawa et al., 1992). The present study is also showing that the amount of amino group in chitosan main chain of PEG–chitosan hybrids was correlated with generation of reactive oxygen species. It was reported that water-insoluble sugar–chitosan hybrids stimulated the PMN cells directly by phagocytosis (Li et al., 1999). Chitosan was also phagocytosed by PMN cells (Minami et al., 1993). We also predict that the direct phagocytosis mechanism was involved in stimulating the PMN cells by water-insoluble PEG–chitosan hybrids. During phagocytosis process, amino group might play an important role in the interaction of PMN and hybrids.

Some interesting reports also informed that the surface modification of chitosan with water-soluble polymer such as poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO), could prevent plasma protein adsorption, platelet adhesion, and thrombus formation by steric repulsion (Amiji & Park, 1995; Hoffman, 1987; Ikada, 1984). Steric repulsion by surface-bound water soluble polymer chains occurs a result of overlapping polymer layers which could lead to loss in configurational entropy because of volume restriction and/or osmotic repulsion between interdigitated polymer chains (Amiji & Park, 1995). Amiji modified chitosan surface with methoxypoly(ethylene glycol) sulfonate (MPEG sulfonate) and showed that surface-immobilized MPEG sulfonate was effective in preventing plasma protein adsorption and platelet adhesion and activation by steric repulsion mechanism (Amiji, 1997). So, the steric hindrance by side PEG chain might also influence the interaction of amino groups in chitosan main chain with PMN cells.

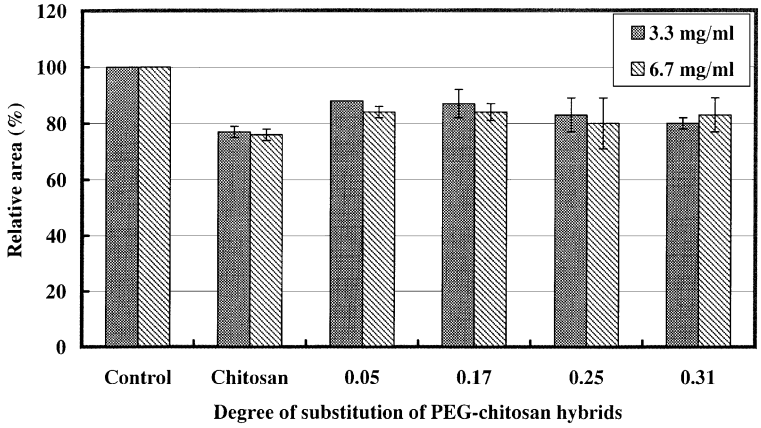


Fig. 3. Effect on complement component C3 activation by water-insoluble PEG–chitosan hybrids and chitosan. Relative area of precipitation rings for chitosan and PEG–chitosan hybrids having different degree of substitution were calculated with respect to the control as 100%. Data are expressed in mean ± SD ($n = 3$). Sample concentration is specified in figure.

3.2. Influence on C3 activation by chitosan–PEG hybrids

Complement is a group of plasma proteins that form the principal effector arm of the humoral immune system and can be activated via two distinct routes, the classical and alternative pathways which are triggered by the presence of foreign materials. The complement component C3 is a major plasma glycoprotein and it plays a central role in the system (Law & Reid, 1995). Recently, we reported that chitosan activated complement component C3 via the alternative pathway (Minami, Suzuki, Okamoto, Fujinaga & Shigemasa, 1998).

In our present study, we compared the influence on activation of C3 by chitosan or water-insoluble PEG–chitosan hybrids, by estimating residual concentration of C3 in the plasma by SRID method. The residual concentration of C3 was expressed in relative area (%) with respect to control (original concentration). Experimental data for water-insoluble PEG–chitosan hybrids having different DS value is shown in Fig. 3. In both cases, for chitosan and PEG–chitosan hybrid, residual C3 in plasma was lower than control and the extents of C3 decreasing were almost same. These results indicate that water-insoluble PEG–chitosan hybrid would be able to activate C3 similar to chitosan. However, no significant influence of the degree of substitution on the C3 activation was observed in our study.

4. Conclusion

Amount of PEG chain and amino group in PEG–chitosan hybrid played important role on its bioactivity. Decrease in amino group content in PEG–chitosan hybrid and increase in the PEG amount decreased the bioactivity. The decrease of amino group in chitosan main chain and increase of the steric hindrance by side PEG chain might reduce the interaction of chitosan with PMN and plasma.

Acknowledgements

This work was partially supported by the Ministry of Education, Science, Sports and Culture of Japan (Grant-in-Aid for Scientific Research on Priority Areas (A) No. 09240103).

References

Aiba, S. (1993). Reactivity of partially *N*-acetylated chitosan in aqueous media. *Makromolekulare Chemie*, *194*, 65–75.

Allen, R. C., Stjernholm, R. L., & Steele, R. H. (1972). Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochemical and Biophysical Research Communications*, *47* (4), 679–684.

Amiji, M. M., & Park, K. (1995). Surface modification of polymeric biomaterials with poly(ethylene oxide), albumin, and heparin for reduced thrombogenicity. In S. L. Cooper, C. H. Bamford & T. Tsuruta, *Polymer biomaterials: in solution, as interfaces, and as solids* (pp. 535–552). Netherlands: VSP.

Amiji, M. M. (1997). Synthesis of anionic poly(ethylene glycol) derivative for chitosan surface modification in blood-contacting applications. *Carbohydrate Polymers*, *32*, 193–199.

Aoi, K., Takasu, A., & Okada, M. (1994). Synthesis of novel chitin derivatives having poly(2-alkyl-2-oxazoline) side chains. *Macromolecular Chemistry and Physics*, *195*, 3844–3855.

Babior, B. M. (1984). Oxidants from phagocytes: agents of defense and destruction. *Blood*, *64* (5), 959–966.

Blair, H. S., Guthrie, J., Law, T., & Turkington, P. (1987). Chitosan and modified chitosan membranes. I. Preparation and characterization. *Journal of Applied Polymer Science*, *33* (2), 641–656.

Dutkiewicz, J., Szosland, L., Kucharska, M., Judkiewicz, L., & Ciszewski, R. (1990). Structure–bioactivity relationship of chitin derivatives. Part I. The effect of solid chitin derivatives on blood coagulation. *Journal of Bioactive and Compatible Polymers*, *5* (3), 293–304.

Dal Pozzo, A., Vanini, L., Fagnoni, M., Guerrini, M., De Benedittis, A., & Muzzarelli, R. A. A. (2000). Preparation and characterization of poly (ethylene glycol)-crosslinked reacetylated chitosans. *Carbohydrate Polymers*, *42*, 201–206.

Harris, J. M., Struck, E. C., Case, M. G., Paley, M. S., Yalpani, M., Alstine, J. M., & Brooks, D. E. (1984). Synthesis and characterization of poly

(ethylene glycol) derivatives. *Journal of Polymer Science, Polymer Chemistry Edition*, *22*, 341–352.

Hoffman, A. S. (1987). Modification of material surfaces to affect how they interact with blood. *Annals of the New York Academy of Sciences*, *516*, 96–101.

Hoffman, A. S., Chen, G., Wu, X., Ding, Z., Kabra, B., Randeri, K., Schiller, M., Ron, E., Peppas, N. A., & Brazel, C. (1997). Graft copolymers of PEO–PPO–PEO triblock polyethers on bioadhesive polymer backbones: synthesis and properties. *Polymer Preprints (American Chemical Society, Division of Polymer Chemistry)*, *38*, 524–525.

Ikada, Y. (1984). Blood-compatible surfaces. *Advances in Polymer Science*, *57*, 103–140.

Imoto, T., & Yanagishita, K. (1971). A simple measurement of lysozyme. *Agricultural and Biological Chemistry*, *35* (7), 1154–1156.

Kurita, K., Yoshida, A., & Koyama, Y. (1988). Studies on chitin 13. New polysaccharide/polypeptide hybrid materials based on chitin and poly(γ-methyl L-glutamate). *Macromolecules*, *21*, 1579–1583.

Law, S. K. A., & Reid, K. B. M. (1995). Complement. In D. Male, *Complement* (2nd ed.) (pp. 1–9). Oxford: Oxford University Press.

Li, X., Morimoto, M., Sashiwa, H., Saimoto, H., Okamoto, Y., Minami, S., & Shigemasa, Y. (1999). Synthesis of chitosan–sugar hybrid and evaluation of its bioactivity. *Polymers for Advanced Technologies*, *10*, 455–458.

Makimura, S., & Sawaki, M. (1992). Evaluation of phagocytic function of canine peripheral polymorphonuclear leucocytes by whole blood chemiluminescence. *Journal of Veterinary Medical Science*, *54* (1), 63–67.

Minami, S., Okamoto, Y., Tanioka, S. I., Sashiwa, H., Saimoto, H., Matsuhashi, A., & Shigemasa, Y. (1993). Effects of chitosan on wound healing. In M. Yalpani, *Carbohydrates and carbohydrate polymers* (pp. 141–152). Mount Prospect Illinois: ATL Press.

Minami, S., Suzuki, Y., Okamoto, Y., Morimoto, M., Sashiwa, H., Saimoto, H., Shigemasa, Y. (1998). Mechanism of complement activation by chitosan. In R. H.Chen & H. C. Chen (Eds.), *Advances in chitin science*, Vol. 3 (pp. 315–322). Keelung: Rita Advertising Co. Ltd.

Minami, S., Suzuki, H., Okamoto, Y., Fujinaga, T., & Shigemasa, Y. (1998). Chitin and chitosan activate complement via the alternative pathway. *Carbohydrate Polymers*, *36*, 151–155.

Murata, J., Saiki, I., Nishimura, S., Nishi, N., Tokura, S., & Azuma, I. (1989). Inhibitory effect of chitin heparinoids on the lung metastasis of B16-BL6 melanoma. *Japanese Journal of Cancer Research*, *80*, 866–872.

Nishimura, S., Nishi, N., Tokura, S., Nishimura, K., & Azuma, I. (1986). Bioactive chitin derivatives. Activation of mouse peritoneal macrophages by *O*-(carboxymethyl)chitins. *Carbohydrate Research*, *146*, 251–258.

Ouchi, T., Nishizawa, H., & Ohya, Y. (1998). Aggregation phenomenon of PEG-grafted chitosan in aqueous solution. *Polymer*, *39* (21), 5171–5175.

Pabst, M. J., & Johnson Jr, R. B. (1980). Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. *Journal of Experimental Medicine*, *151*, 101–114.

Saito, H., Wu, X., Harris, J. M., & Hoffman, A. S. (1997). Graft copolymers of poly(ethylene glycol) (PEG) and chitosan. *Macromolecular Rapid Communication*, *18*, 547–550.

Shigemasa, Y., & Minami, S. (1995). Applications of chitin and chitosan for biomaterials. *Biotechnology and Genetic Engineering Reviews*, *13*, 383–420.

Shigemasa, Y., Matsuura, H., Sashiwa, H., & Saimoto, H. (1996). Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules*, *18*, 237–242.

Sugimoto, M., Morimoto, M., Sashiwa, H., Saimoto, H., & Shigemasa, Y. (1998). Preparation and characterization of water-soluble chitin and chitosan derivatives. *Carbohydrate Polymers*, *36*, 49–59.

Sugimoto, M., & Shigemasa, Y. (1998). Interaction between chitin/chitosan–PEG hybrid and acrylic emulsion. *Chitin and Chitosan Research*, *4* (2), 85–96.

Tanigawa, T., Tanaka, Y., Sashiwa, H., Suzuki, H., Saimoto, H., & Shigemasa, Y. (1992). Various biological effects of chitin derivatives. In C. J. Brine, P. A. Sandford & J. P. Zikakis, *Advances in chitin and chitosan* (pp. 206–215). London and New York: Elsevier.

Terada, N., Morimoto, M., Saimoto, H., Okamoto, Y., Minami, S., & Shigemasa, Y. (1999). Synthesis of water-soluble oxidized chitosan derivatives and their biological activity. *Chemistry Letters*, 1285–1286.

Trush, M. A., Wilson, M. E., & Dyke, K. V. (1978). The generation of chemiluminescence (CL) by phagocytic cells. In M. A. DeLuca, *Methods in enzymology*, Vol. 57 (pp. 462–494). New York: Academic Press.

Usami, Y., Okamoto, Y., Takayama, T., Shigemasa, Y., & Minami, S. (1998). Effect of *N*-acetyl-D-glucosamine and D-glucosamine oligomers on canine polymorphonuclear cells in vitro. *Carbohydrate Polymers*, *36*, 137–141.

Vaerman, J. (1981). Single radial immunodiffusion. In J. J. Langone & H. V. Vunakis, *Methods in enzymology*, Vol. 73 (pp. 291–305). New York: Academic Press.

Yalpani, M., Marchessault, R. H., Morin, F. G., & Monasterios, C. J. (1991). Synthesis of poly(3-hydroxyalkanoate) (PHA) conjugates: PHA-carbohydrate and PHA-synthetic polymer conjugates. *Macromolecules*, *24*, 6046–6049.

Yalpani, M., Johnson, F., & Robinson, L. E. (1992). Antimicrobial activity of some chitosan derivatives. In C. J. Brine, P. A. Sandford & J. P. Zikakis, *Advances in chitin and chitosan* (pp. 543–548). London and New York: Elsevier.