Novel Water-Soluble Photosensitizers from Chitosan

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Received May 10, 2006; Revised Manuscript Received October 30, 2006

Novel water-soluble polymeric photosensitizers based on the natural polymer chitosan were synthesized and studied. The modified chitosans contain covalently attached Rose Bengal. The polymers absorb light from the visible spectral region and generate singlet oxygen. They can serve as environmentally friendly, biodegradable polymeric photosensitizers, which can use light from a visible spectral region to initiate photooxidation of organic compounds in water.

Introduction

Water-soluble polymers that contain photoactive groups have attracted considerable attention as potential photosensitizers.[1,2] Due to environmental concerns we have been interested in developing such systems based on natural polymers.[3] In this paper we present results on the synthesis and properties of the photosensitizers based on chitosan (CH). CH was chosen as it is a derivative of chitin, one of the most abundant natural polymers in the biosphere. Chitin is the main component of the exoskeletons of marine crustaceans (e.g., shrimps, crabs, krill), which are available in large amounts as a byproduct of food processing. CH, which can be obtained by N-deacetylation of chitin, is a polysaccharide that possesses many useful properties and has found many practical applications. Due to its biodegradability, biocompatibility, nontoxicity, antibacterial,[4] and antiviral[5] properties it is currently receiving a great deal of interest for medical, pharmaceutical, and agricultural applications. In this work CH chains were modified by covalent attachment of the xanthene dye Rose Bengal (RB). RB is a well-known photosensitizer for singlet oxygen reactions.[6,7] It has many advantages; it is a water-soluble, nontoxic dye, and it absorbs light in the visible spectral region. Its practical application is, however, strongly limited by the fact that it tends to aggregate in aqueous solution. The process of separation of such a photosensitizer from the reaction mixture (when the reaction is completed) is also very difficult. These problems can be diminished by attachment of RB to the polymer chain.

The main aim of our current studies was to obtain photosensitizers that could be applied to photosensitize oxidation reactions of various organic molecules in water.

Experimental Section

Apparatus. The ^{1}H NMR spectra of the polymers were measured in D_2O/CF_3COOD solution using a Bruker AMX 500 spectrometer. The IR spectra were recorded in KBr pellets using a Bruker IFS 48 spectrometer (Bruker, Rheinstetten, Germany). The dynamic light scattering (DLS) measurements were performed using Malvern Nano ZS light-scattering apparatus (Malvern Instrument Ltd., Worcestershire, U. K.). Atomic force microscopy (AFM) images were obtained with a MultiMode scanning probe microscope with a NanoScope IVA controller working in tapping mode equipped with a silicon cantilever (Digital Instruments, Santa Barbara, CA). The UV−vis spectra of the samples were obtained using a HP 8452A diode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The steady-state fluorescence spectra were obtained using a SLM Aminco 8100 spectrofluorimeter (SLM, Rochester, NY) set up in the L-type geometry. The gel permeation chromatography (GPC) analyses of the polymers were carried out using a Waters GPC system (Waters, Milford, MA) equipped with an Ultrahydrogel linear 8 μm column. Detection was done using a Waters 2410 refractive index (RI) detector, a Waters 474 scanning fluorescence detector, and a Waters 2996 photodiode-array detector. A 0.25 M acetic acid/0.25 M sodium acetate solution was used as the mobile phase at flow rate of 0.5 mL/min. The viscosities of the polymer solutions were measured using an Ubbelhode capillary viscometer equipped with an electronic time-measuring unit ViscoClock (Schott, Mainz, Germany).

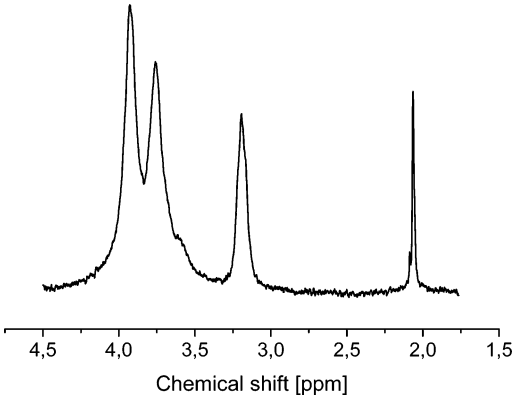

Figure 1. ^{1}H NMR spectrum of chitosan.

Irradiation of the samples was performed with a mercury lamp (150 W) equipped with a 475 nm cutoff filter. During the irradiation, the solutions were oxygenated by bubbling with oxygen and were mixed with a magnetic stirring bar.

Materials. A chitosan (CH) sample (a shrimp-based product) was obtained from Novachem Limited of Dartmouth, Nova Scotia, Canada. The viscosity and weight average molecular weights, M_v and M_w, of the polymer were determined to be 1300 and 1230 kDa based on the Mark−Houwink−Sakurada equation using the literature values[8] of $K = 1.57 \times 10^{-4}$ mL/g, $\alpha = 0.79$, and $q_{MHS} = 0.95$. Rose Bengal (RB, Aldrich, certified grade) was used as received. Anthracene-2-sulfonic acid, sodium salt (ANS), was prepared by reduction of anthraquinone-2-sulfonic acid, sodium salt (Aldrich), with Zn dust in the presence of NH_4OH. Acetonitrile (POCH Gliwice, HPLC grade), tetrahydrofuran (THF, Aldrich, spectrograde), methanol (POCH Gliwice, HPLC grade), D-(+)-glucosamine hydrochloride (GA, Aldrich, >99%), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma,

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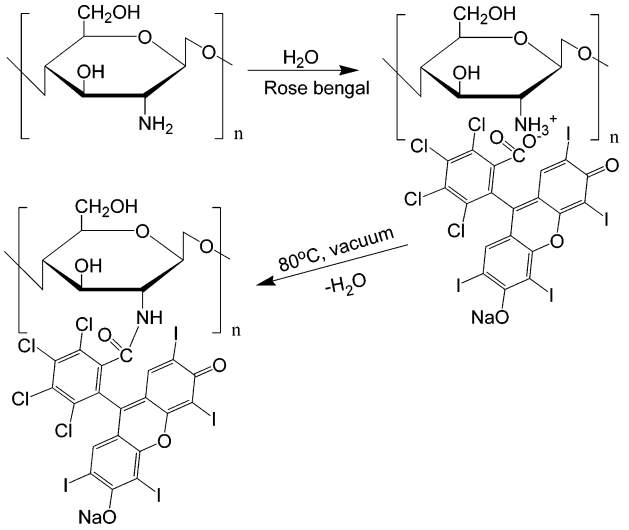
10.1021/bm060454+ CCC: $37.00
Published on Web 01/12/2007

Scheme 1. Reaction between CH and RB



protein sequencing grade), 1-hydroxy-2,5-pyrrolidinedione (NHS, Aldrich, >97%), acetic acid (AA, POCH, Gliwice, 99,98%), and sodium acetate (NaAc, POCH Gliwice, analytically pure) were used without further purification. The aqueous solutions were prepared using double-distilled water.

Acetylation Degree of CH. The degree of acetylation (DA) of CH (12%) was determined by ^{1}H NMR and elemental analysis (EA) methods. The NMR spectrum (Figure 1) was used to find the degree of acetylation using the following formula

$$\mathrm{DA} = \frac{^1/_3(\text{surface area at 2.1 ppm})}{(\text{surface area at 3.2 ppm}) + {}^1/_3(\text{surface area at 2.1 ppm})} \times 100\% \quad (1)$$

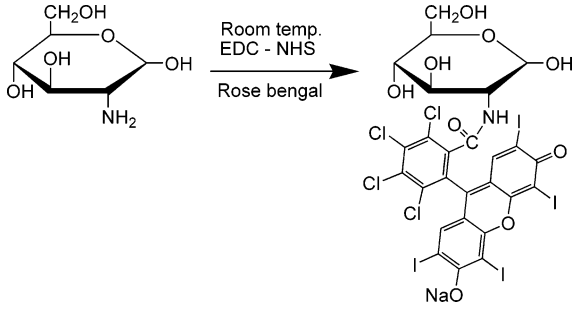
A similar value of DA was obtained from EA

$$\mathrm{DA} = \frac{8.69 - \%\ \text{nitrogen content}}{8.69 - 6.89} \times 100\% \quad (2)$$

Synthesis of the Polymers (CHRB). Two CHRB polymers characterized by different degrees of substitution with RB were obtained. The polymers were synthesized by dehydration of the chitosan amino RB salt (Scheme 1). The general procedure was as follows: CH (0.5 g) was dissolved in 1% AA aqueous solution (25 mL) and was stirred at room temperature for 12 h. Then a solution of RB in MeOH (0.27 or 0.027 g, respectively) was added dropwise over a period of ca. 30 min. The mixture was intensively stirred using a magnetic stirring bar for 24 h. After the reaction was completed the mixture was kept for 12 h under vacuum at 80 °C to dehydrate the CH salt formed and obtain an amide linkage between CH and RB. The product was rinsed with methanol several times and then exhaustively dialyzed (Sigma, cellulose tubing, cutoff 12 000−14 000 g/mol) against water (basic and acid, alternately) for a week to remove nonbound RB. The dialysis was finished when no RB residues were detected by measurement of the UV−vis spectrum of the dialysate. The polymers were subsequently freeze-dried. The content of RB in the polymers was found to be 0.013 mol % for CHRB1 and 0.35 mol % for CHRB2 (molar ratio with respect to the glucosamine unit of CH).

Synthesis of the Model Compound (GARB). RB was attached to GA in the reaction similar to that in which CHRB polymers were obtained (Scheme 2). To a solution of GA (0.2 g in deionized water) a solution of RB (1 g in MeOH) was added. The mixture was stirred for several hours, and then EDC (0.18 g) and NHS (0.05 g) were added. The reaction was carried out at room temperature for 24 h. Then the product was filtered out, rinsed with methanol and water, and freeze-dried.

Scheme 2. Reaction Leading to GARB



Results and Discussion

Properties of CHRB Polymers. The CHRB polymers were obtained as a pink powder. They were soluble in water at pH values lower than 6; the polymer with a lower content of RB was soluble without limitation while the polymer with a higher content of dye was soluble up to concentration 1 g/L. The polymers were characterized by elemental analysis, DLS, AFM, viscosimetry, spectroscopic methods (^{1}H NMR, Fourier transform infrared (FTIR), and absorption and emission in the UV−vis spectral region) and by GPC.

The ^{1}H NMR spectra did not show signals characteristic of RB chromophores (at 7.3 ppm) because their content was too low, and they were used only to find the DA of chitosan.

FTIR spectra of the substituted polymers showed bands that could be assigned to the amide bonds between CH and RB (Figure 2). There are two characteristic peaks at 1655 (amide I, carbonyl stretching vibration) and 1595 cm^{-1} that correspond to N-acetylated units and free amino groups, respectively. Unfortunately, the bands that come from amide bonds of acetyl groups in chitosan and those from RB are in the same area in the IR spectrum. But the ratio of intensities at 1595−1655 cm^{-1} is higher in the FTIR spectrum of CHRB2. Moreover, the peak of the amino groups of CHRB2 is shifted to lower frequencies, which suggests a decrease in association via hydrogen bonds.[9] In addition, one may notice that FTIR spectra do not show a band characteristic of ester groups (ca. 1730 cm^{-1}) and that fact may be a confirmation that RB units are attached via amide bonds. However, the reaction between the hydroxylic groups of CH and the carboxylic groups of RB is possible and may occur to some extent.[10]

The modified CHs were also characterized by GPC. The GPC chromatogram for the starting material, CH, displayed one intensive peak at a retention time of 13.45 min, which is shifted to a slightly longer retention time (13.90 min) for CHRB2

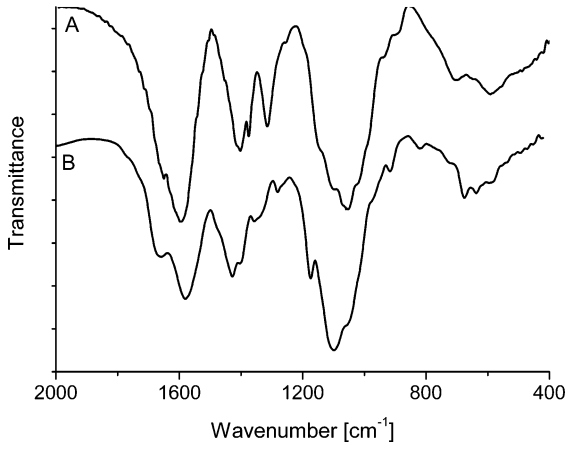


Figure 2. FTIR spectra of (A) CH and (B) CHRB2.

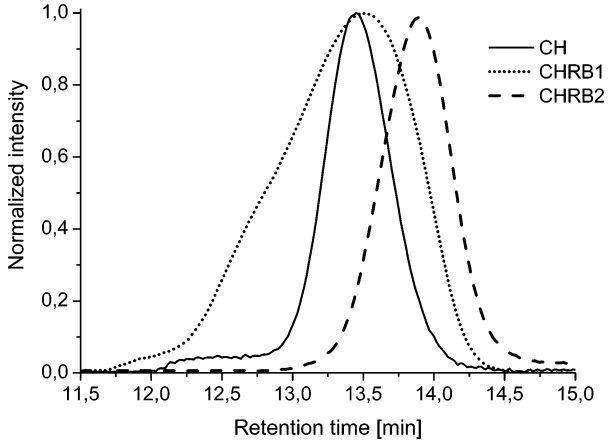


Figure 3. Normalized GPC traces of CH, CHRB1, and CHRB2 (RI detector, eluent 0.25 M AA and 0.25 M NaAc, pH 4.7, as recommended in the literature[8]).

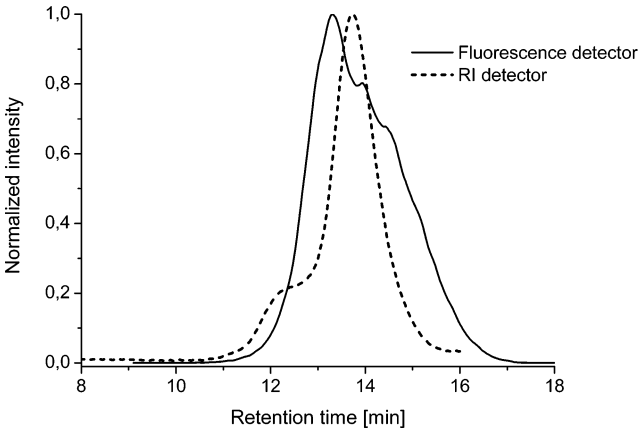


Figure 4. Normalized GPC traces of CHRB2 (RI and fluorescence detectors, eluent 0.25 M AA and 0.25 M NaAc, pH 4.7, as recommended in the literature[8]).

(Figure 3). That effect can be explained by taking into account a decrease in the hydrodynamic volume of CH while substituted with hydrophobic RB units. The effect is not very pronounced because the chitosan molecules are quite stiff. The GPC trace for CHRB1 shows almost the same retention time as CH, but the peak is visibly broadened. That suggests that the RB present in the polymer chains induces considerable changes in their conformation.

To confirm covalent bond formation between RB chromophores and CH the GPC technique was applied. The GPC chromatograms for CHRB2 were recorded using RI and fluorescence detectors simultaneously. Data shown in Figure 4 indicate that both traces overlap, which clearly indicate RB is a part of the polymer chain and not a separate molecule.

The DLS (Figure 5) confirms the data obtained from GPC. The hydrodynamic diameters for CH, CHRB1, and CHRB2 are 25, 50, and 20 nm, respectively.

To determine the M_v of CH and compare the intrinsic viscosities of the studied materials viscometric measurements were performed. Reduced viscosities versus concentrations are depicted in (Figure 6). The results show that the intrinsic viscosities of CH and CHRB1 are quite similar while the value of η for CHRB2 is almost 2 times lower that that for CH. That observation fits the data obtained for CH and CHRB2 from DLS and GPC. Comparing the results obtained from DLS for the CHRB1 polymer one could expect that the intrinsic viscosity should be higher than that measured for CH and CHRB2

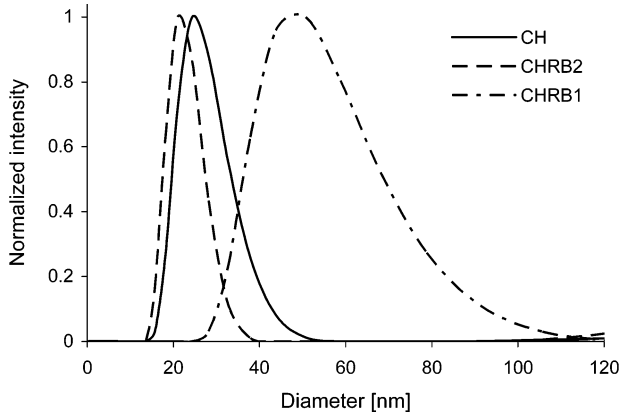


Figure 5. Distribution profiles of the hydrodynamic diameters measured by dynamic light scattering of CH, CHRB1, and CHRB2.

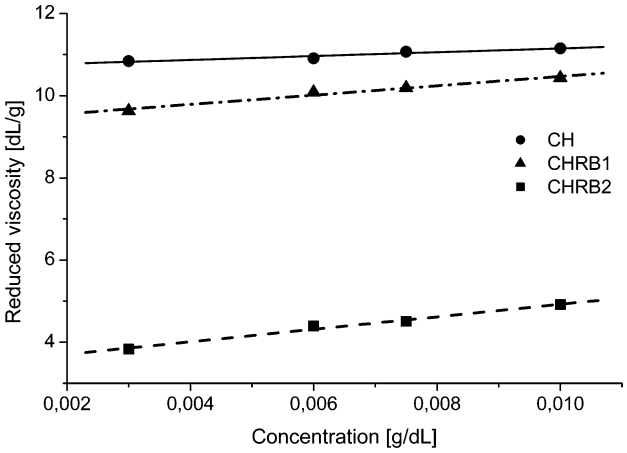


Figure 6. Reduced viscosity versus concentration for CH, CHRB1, and CHRB2.

(Figures 5 and 6). This phenomenon is connected with the specificity of that research technique. DLS is a static method that allows observation of the polymeric aggregates formed in aqueous solution. However, viscosimetry is a dynamic method, and during a flow of the solution through the capillary the polymeric aggregates could be destroyed.

The AFM measurements supported the previous results obtained with other techniques. The AFM images captured for CH, CHRB1, and CHRB2 polymers are depicted below (Figures 7A, 7B, and 7C).

The AFM picture of CH shows that it forms rather isolated domains that have a mean diameter of approximately 30 nm (Figure 7A). The hydrogen bonds are believed to play an essential role in organizing the macromolecules of CH. The film structures of CHRB1 and CHRB2 are quite different. The CHRB2 chains form smaller domains than CH, and CHRB1 chains form larger domains than CH. The introduction of RB molecules to CH leads to the partial elimination of hydrogen bonds, which effects the hydrodynamic volume of the polymer chain in solution and the organization on a mica support. The higher content of RB causes stronger hydrophobic interactions between macromolecules of CHRB2 (Figure 7C), and the grain diameters are small (15−25 nm). As for CHRB1, the film consists of complex structures with sizes of approximately 50−60 nm (Figure 7B). It is very interesting to observe how even a very low content of the dye changes the hydrodynamic properties and the organization of the macromolecules in a film.

Electronic absorption spectra for the polymers (shown for CHRB2 in Figure 8) displayed bands characteristic of RB

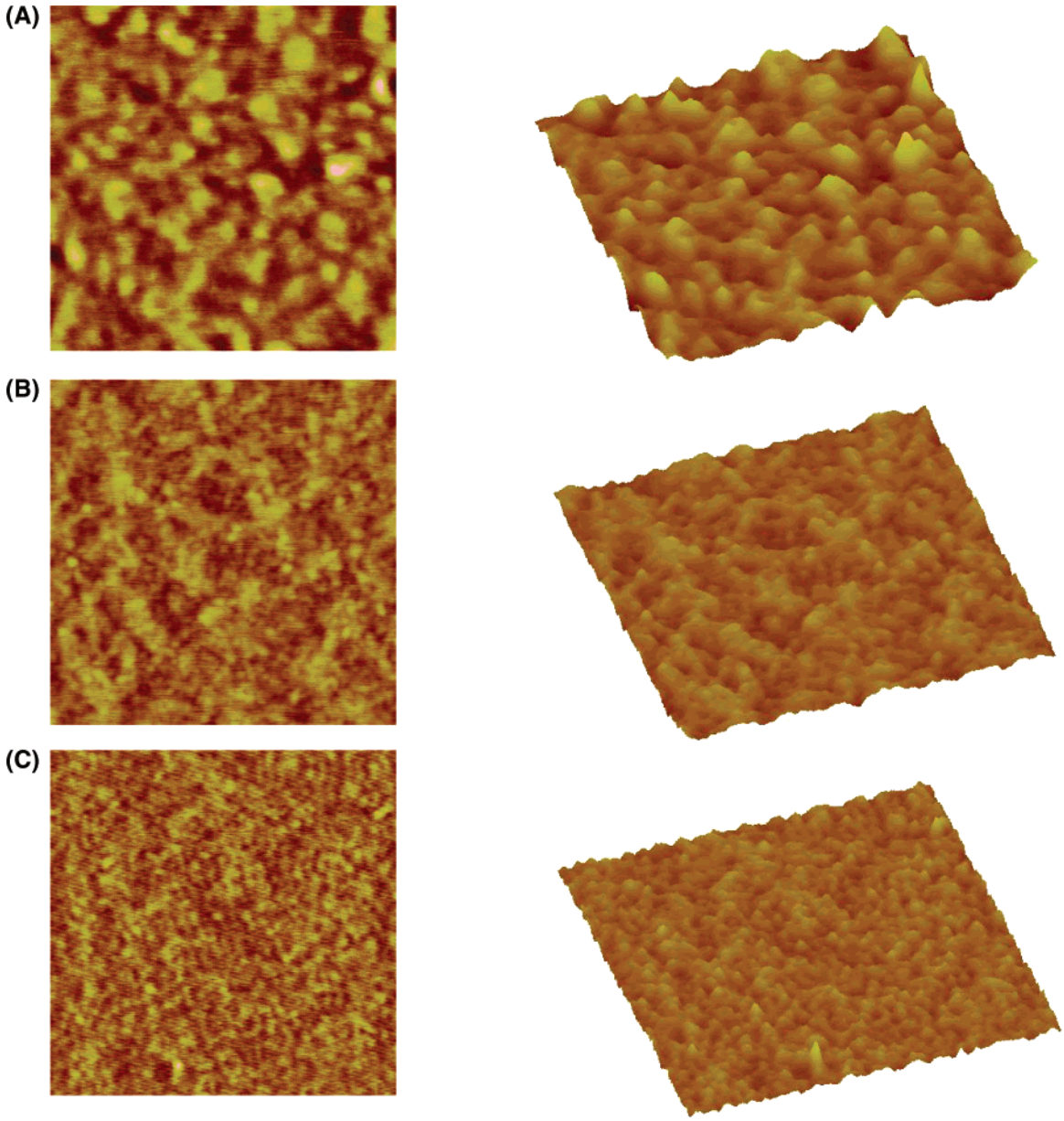


Figure 7. AFM images of (A) CH, (B) CHRB1, and (C) CHRB2. All pictures are 500 nm × 500 nm with the height image shown on the left and the three-dimensional image shown on the right. The films were prepared by immersion of mica substrate into solution (c = 0.1 g/L) of a polymer.

chromophores. However, the absorption bands for CHRB in water are broader and red-shifted with respect to those of molecular RB. That confirms that the RB chromophores are attached to the polymer chain.

There is, however, a problem with their quantitative characterization. Due to the fact that dye chromophores are attached to the CH chain via amide linkages one cannot make a direct comparison of their spectroscopic properties with these characteristics for the free RB molecules. It is known that the electronic absorption spectra of RB are strongly dependent on the degree of ionization. Thus, the model compound, GARB, D-glucosamine with RB chromophores attached via amide bonds, was synthesized and characterized by spectroscopic techniques. Although GARB is structurally a perfect model for the polymeric dye chromophores present in CHRB, there is, however, a solubility problem. Unfortunately, GARB is not soluble in water. It is very soluble in THF. For our purposes we have measured its absorption spectra in several mixtures of water and acetonitrile (Figure 9A). As expected, the shape, the position, and the intensity of the GARB spectrum are strongly dependent on the composition of the solvent (Figure 9B). That can be explained by taking into account the aggregation and the ionization of the compound in the solvents used. In pure acetonitrile GARB is soluble, but the chromophores are not ionized. When water is added, the intensities of the absorbance peaks increase steadily (in water ionization of the dye is possible) up to a certain point (1:1 (v/v)). Up to that point the effect of ionization is stronger than the aggregation tendency of GARB. The subsequent increase of water content results in a decrease of the absorption intensity. In pure water one can observe the broad, structureless spectrum of GARB. The changes in solvent composition also lead to shifts of the maximum absorbance. By comparison of the shapes of the spectra and the ratios of absorbance values at the absorption maxima, it was decided that the composition of solvent 1:14 (v/v) of acetonitrile to water reflected the best situation for dye chromophores of CHRB in aqueous polymer solutions. Thus the value of the molar extinction coefficient ϵ at λ = 560 nm

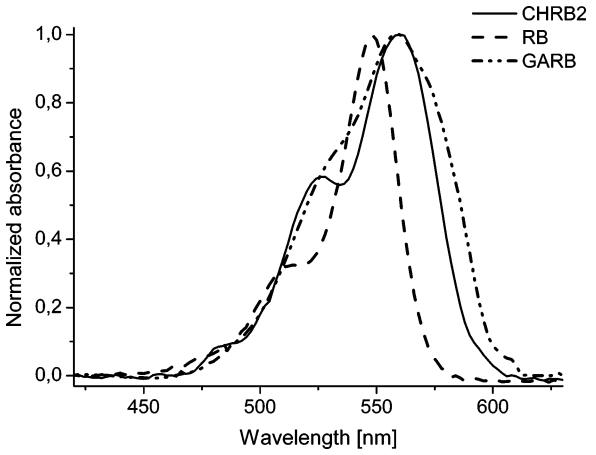


Figure 8. Normalized electronic absorption spectra of aqueous solutions of RB ($c = 5 \times 10^{-6}$ M), CHRB2 ($c_{pol} = 0.1$ g/L, pH = 3.5), and GARB ($c = 5 \times 10^{-6}$ M).

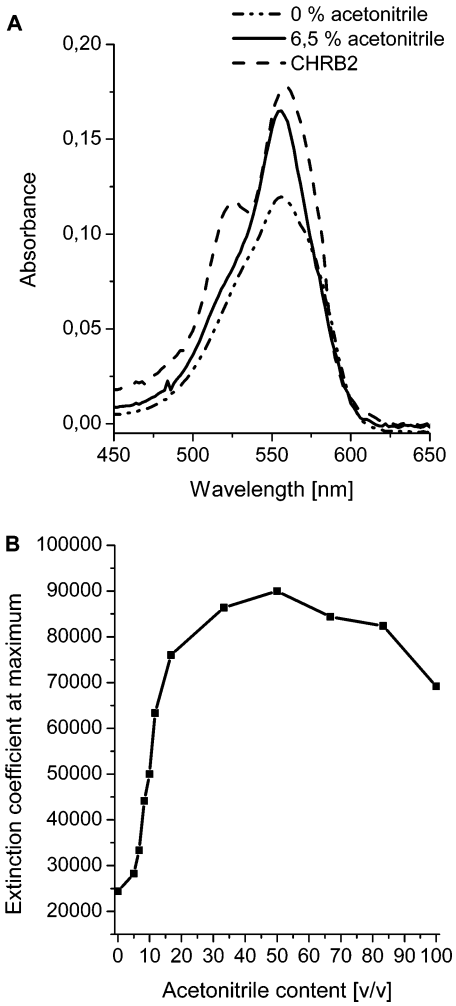


Figure 9. (A) Absorption spectra of GARB in the mixture of acetonitrile/water ($c_{GARB} = 5 \times 10^{-6}$ M). (B) Dependence of GARB ϵ at maximum versus content of acetonitrile in water (% (v/v)).

was determined for GARB in that solution to be 45 000 cm^{-1} M^{-1} and was used to characterize CHRB polymers.

Comparison of the absorption and excitation spectra of CHRB2 in aqueous solution indicates that the interactions between RB chromophores in the ground state can be neglected (no additional bands in the excitation spectrum were observed) (Figure 10). The steady-state emission spectrum is an almost perfect mirror image of the excitation spectrum.

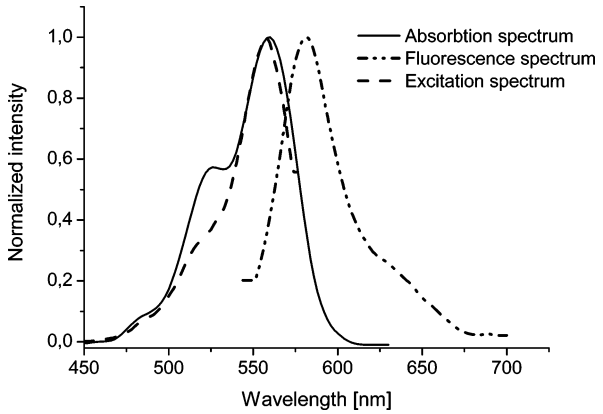


Figure 10. Electronic absorption, excitation, and emission spectra of CHRB2 in water ($c_{pol} = 0.1$ g/L, pH = 3.5).

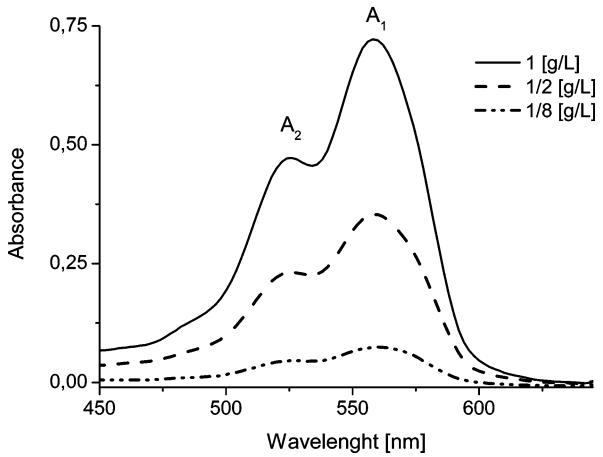


Figure 11. Electronic absorption spectra of CHRB2 in water at different concentrations.

Thus one can conclude that aggregation of the RB chromophores is negligible. That conclusion is supported by the findings that the shape of the absorption spectrum and the ratio of the absorbance at the absorption maxima (A_1/A_2)[11] are not dependent on polymer concentration in the concentration range studied (1−0.1 g/L) and that the ratio is equal to 1.6 (Figure 11).

Determination of the Quantum Yields of Singlet Oxygen Formation. The quantum yield of singlet oxygen formation by the RB chromophores covalently attached to the CH chains (Φ_{P-RB}) was determined in aqueous solution by using the relative actinometry method.[12] That method requires determination of the rates of oxidation of a singlet oxygen acceptor photosensitized by molecular RB (V_{RB}) and by RB bound to the polymer chain (V_{P-RB}). The reactions must be conducted at an acceptor concentration that is high enough to ensure that the reaction is zero order with respect to the acceptor. Under these conditions, the quantum yield of singlet oxygen formation by the RB chromophores attached to the CH chain can be calculated using the following equation

$$\Phi_{P-RB} = \Phi_{RB} \frac{V_{P-RB}}{V_{RB}} \quad (3)$$

where Φ_{RB} is the quantum yield of singlet oxygen formation by free RB molecules in water, which is equal to 0.76.[13]

The quantum yields of singlet oxygen formation were determined for both polymers studied at the same total concentration of RB chromophores ($c = 2 \times 10^{-5}$ M). ANS

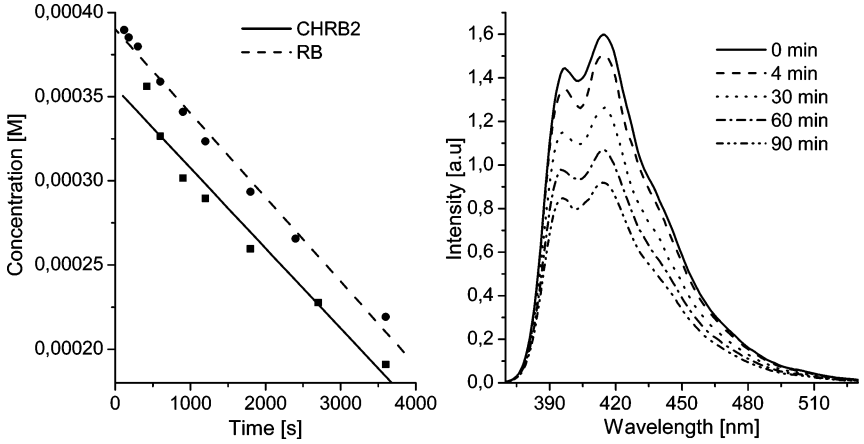


Figure 12. Changes in ANS concentration during oxidation photosensitized in an aqueous solution of RB and CHRB2.

Table 1. Rate Constants of Photosensitized Oxidation of ANS (k) and Quantum Yields of Singlet Oxygen Formation (Φ)

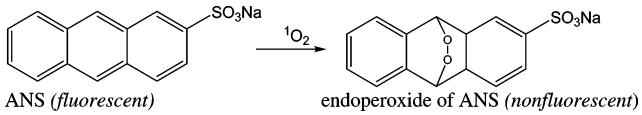
polymer	Φ	$k \times 10^8$ (mol/s)
CHRB1	0.71 ± 0.05	4.5 ± 0.5
CHRB2	0.83 ± 0.08	5.0 ± 0.5
RB	0.76	5.0

was used as the singlet oxygen acceptor. The solution of ANS with an initial concentration equal to 4×10^{-4} M and the photosensitizer were irradiated with light that was absorbed only by RB chromophores (a cutoff filter 475 nm was used), and the changes in ANS concentration were determined from the changes in fluorescence intensity (Figure 12). The reaction can be described by the following scheme:

1. Singlet oxygen generation

$$RB \xrightarrow{h\nu} {}^1RB^* \xrightarrow{ISC} {}^3RB^*$$
$${}^3RB^* + {}^3O_2 \longrightarrow RB + {}^1O_2$$

2. Photosensitized reaction



The experimentally determined values of the rate constants of ANS oxidation and calculated quantum yields of singlet oxygen formation by RB and CHRB1 and CHRB2 were given in Table 1. The value of rate constant of $(5 \pm 0.5)\ 10^{-8}$ mol/s obtained for RB is in very good agreement with that obtained earlier by Schaap et al.[12] The rates of ANS oxidation and quantum yields of singlet oxygen formation obtained where polymers were used as photosensitizers are very similar to that characteristics for free RB. Thus, one can conclude that attachment RB to the polymeric chains did not decrease the photosensitizing activity of chromophores. That finding corresponds well with the observation that RB chromophores attached to CH chains do not undergo considerable aggregation.

Conclusions

Novel photoactive water-soluble modified chitosans were prepared. The polymers varied in their content of RB chromophores. The polymers absorb light from a visible spectral region. Although the content of RB was low the polymers are very efficient generators of singlet oxygen. Thus, these polymers can find application as environmentally friendly photosensitizers for various oxidation reactions carried out in water.

Acknowledgment. The authors are grateful to the Polish Ministry of Education and Science for financial support (Grants Nos. PBZ-KBN-070/T09/2001/14 and 3 T08E 007 30).

References and Notes

(1) Nowakowska, M.; Guillet, J. E. *Chem. Br.* **1991**, *27*, 327.
(2) Nowakowska, M.; Sterzel, M.; Szczubiałka, K.; Guillet, J. E. *Macromol. Rapid Commun.* **2002**, *23*, 972.
(3) Nowakowska, M.; Zapotoczny, S.; Sterzel, M.; Kot, E. *Biomacromolecules* **2004**, *5*, 1009.
(4) Tsai, G. J.; Su, W. H. *J. Food Prot.* **1999**, *62*, 239.
(5) Chirkov, S. N. *Appl. Biochem. Microbiol.* **2002**, *38*, 5.
(6) Nowakowska, M.; Suster E.; Guillet, J. E. *J. Photochem. Photobiol., A* **1994**, *80*, 369.
(7) Nowakowska, M.; Kępczyński, M. *J. Photochem. Photobiol., A* **1998**, *116*, 251.
(8) Kasaai, M. R.; Arul, J.; Charlet, G. *J. Polym. Sci., Part B: Polym. Phys.* **2000**, *38*, 2591.
(9) Silverstein, R. M; Bassler, G.C. *Spectrometric Identification of Organic Compounds*; John Wiley and Sons: New York, 1981.
(10) Qu, X.; Wirsén, A.; Albertsson, A.-C. *J. Appl. Polym. Sci.* **1999**, *74*, 3193.
(11) Neckers, D. C. *J. Photochem. Photobiol., A* **1989**, *47*, 1.
(12) Schaap, A. P.; Thayer, A. L.; Blossey, E. C.; Neckers, D. C. *J. Am. Chem. Soc.* **1974**, *97*, 3741.
(13) Stevens, B.; Ors, J. A.; Pinsky, M. L. *Chem. Phys. Lett.* ***1974***, *27*, 157.

BM060454+