ENZYMATICALLY CATALYZED GENERATION OF ELECTRONICALLY EXCITED STATES IN A SYNTHETIC COPOLYMER WITH A SCHIFF BASE IN PENDANT GROUPS*

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The copolymer of *N*-(2-hydroxypropyl)methacrylamide and *N*-[*N*-(*N*-methacryloylglycyl)glycyl]-hexane-1,6-diamine (17.6 mole %) was prepared as a model compound for investigation of the mechanism of enzymatically catalyzed generation of electronically excited states in proteins. The amino end groups of the copolymer side chains were transformed into a Schiff base by reaction with glycolaldehyde. The modified copolymer was characterized by its ultraviolet, visible and fluorescence spectra. The enzymatically catalyzed oxidation of the modified copolymer was carried out in a phosphate buffer at pH 7 using horseradish peroxidase as the enzyme. The generation of electronically excited states was demonstrated by chemiluminescence measurement and by a spectroscopic procedure based on transfer of the excitation energy to bilirubin.

Enzyme catalyzed generation of electronically excited states is of considerable interest particularly in view of its presumed role in undesirable pathological processes, such as peroxidation of membrane lipids, gene mutation and oxidation of proteins. Such processes are often linked to the oxidative stress of the organism, aterosclerosis, bad compensated diabetes mellitus and several other diseases[1–3].

In laboratories, the generation of excited states has been studied in aerated aqueous solutions of 2-methylpropanal, indole-2-acetic acid or a Schiff base (SB) as the substrate and a dioxygenase (mostly horseradish peroxidase, HRP) as the enzyme[1,4,5]. The reactions of SB seem to be of greatest importance because SB can easy be formed in vivo by condensation of amino groups of the proteins with carbonyl compounds, saccharides in particular[6]. The oxidation of SB is assumed to proceed via the 1,2-dioxetane ring[7,8] according to reaction (*A*).

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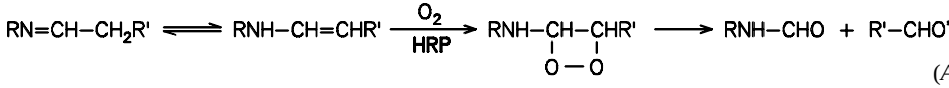
Mechanistic study of the enzymatically catalyzed generation of excited states in proteins is complicated by difficulties encountered during transformation of the free amino groups into SB, because not all amino groups in proteins are accessible for carbonyl compounds. The preparation of suitable model compounds avoiding this complication is therefore desirable. In this paper, we report on the preparation of a possible model compound – a copolymer bearing easily accessible amino groups at the ends of its flexible side chains, on the transformation of the amino groups into SB, and on the generation of electronically excited states in the modified copolymer induced by HRP.



(*A*)

EXPERIMENTAL

Methods

Molecular weights of the polymers were determined by the SEC method (size exclusion chromatography) using a column packed with a mixture of Sepharose 4B and 6B (1 : 1), a light scattering detector, and 0.05 M Tris–acetate buffer pH 8 containing 0.5 M NaCl as the mobile phase (0.5 cm^3 min^{-1}). The column was calibrated using samples of poly[*N*-(2-hydroxypropyl)methacrylamide], and the molecular-weight averages were calculated by the routine procedure without any data correction for axial dispersion. Melting points were determined on a Kofler block. The amino group content in the copolymer was determined by the ninhydrine method[9]. UV-VIS spectra were recorded on a Specord M-40 spectrometer (Zeiss, Jena) at 20 °C. The differential absorption spectra method was employed to monitor the transfer of the excitation energy to bilirubin. Fluorescence spectra were recorded on a Perkin–Elmer LS 5B spectrofluorimeter. Chemiluminescence was measured using the detection unit of a Beckman LS 6000 SE liquid scintillation counter in the coincidence mode.

Chemicals

Acetone and methanol of reagent grade purity (Lachema Brno, The Czech Republic) and chemicals obtained from Fluka, Switzerland, viz. acetonitrile (99.5%), 1-amino-2-propanol (93%), azobis(isobutyronitrile), AIBN (98%), 1,3-dicyclohexylcarbodiimide (99%), *N*,*N*-dimethylformamide (99%), 2,5-di-*tert*-butylhydroquinone (97%), glycylglycine (99.5%), methacryloyl chloride (97%), *p*-nitrophenol (99%), *N*-*tert*-butyloxycarbonyl-1,6-diaminohexane hydrochloride (97%), octylpyrocatechine (98%), and triethylamine (99.5%) were used without further purification. Methacryloyl chloride (b.p. 97 – 100 °C) and acetone (b.p. 55 – 56 °C) were purified by distillation at ambient pressure. Methanol was dried with calcium hydride and distilled. *N*,*N*-Dimethylformamide was purified by vacuum distillation (b.p. 73 – 76 °C).

N-(2-Hydroxypropyl)methacrylamide (*I*)

A solution of methacryloyl chloride (100 g, 0.96 mol) in acetonitrile (406 ml) stabilized with 2,5-di-*tert*-butylhydroquinone (4 mg) was added to a stirred solution of 1-amino-2-propanol (144 g, 1.92 mol) in acetonitrile (376 ml) at −15 °C in 15 min. The mixture was warmed up to 20 °C and stirred for another 15 min, and the precipitated 1-amino-2-propanol hydrochloride was filtered off. The product was isolated by cooling the solution to −40 °C, purified by crystallization from the methanol–diethyl ether 1 : 3 mixture and recrystallization from acetone. Yield 52 g (38%), m.p. 68 – 69 °C (ref.[10], m.p. 65 – 68 °C).

N-[*N*-(*N*-Methacryloylglycyl)glycyl]-*N*′-*tert*-butyloxycarbonylhexane-1,6-diamine (*II*)

Compound *II* was prepared by a modification of the procedure developed in ref.[11] for the synthesis of derivatives of *N*-methacryloylglycylglycine (*III*). Methacryloyl chloride (8.7 g, 83 mmol) in dichloromethane (20 ml) was subjected to the Schotten–Baumann reaction with glycylglycine (11.7 g, 89 mmol) in 15% NaOH (50 ml, 188 mmol) to yield 11.3 g (68%, 57 mmol) of *III*, which was transformed into the corresponding *p*-nitrophenyl ester (*IV*) by reaction with *p*-nitrophenol (8.6 g, 62 mmol) in DMF (100 ml) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (12.8 g, 61 mmol). Subsequently, triethylamine (1.3 ml, 9.5 mmol) divided into three equivalent portions was added (in 10 min intervals) to a stirred solution of *IV* (3 g, 9.3 mmol) and *N*-*tert*-butyloxycarbonyl-1,6-diaminohexane hydrochloride (2.4 g, 9.5 mmol) in DMF (15 ml), and the mixture was stirred at 20 °C for another 3 h. The precipitated triethylamine hydrochloride was filtered off, the filtrate was evaporated in a vacuum, and the crude compound *II* was triturated with diethyl ether and dissolved in chloroform (15 ml). The solution was extracted with water (3 × 10 ml), dried with $MgSO_4$, and evaporated. The residue was recrystallized from chloroform–diethyl ether to yield 1.98 g (54%) of *II*, m.p. 111 °C, TLC (silica gel, Merck $60F_{254}$): R_F = 0.61, methanol. For $C_{19}H_{34}O_5N_4$ (398.5) calculated: 57.27% C, 8.60% H, 14.06% N; found: 57.37% C, 8.80% H, 13.97% N.

Poly[*N*-(2-hydroxypropyl)methacrylamide]

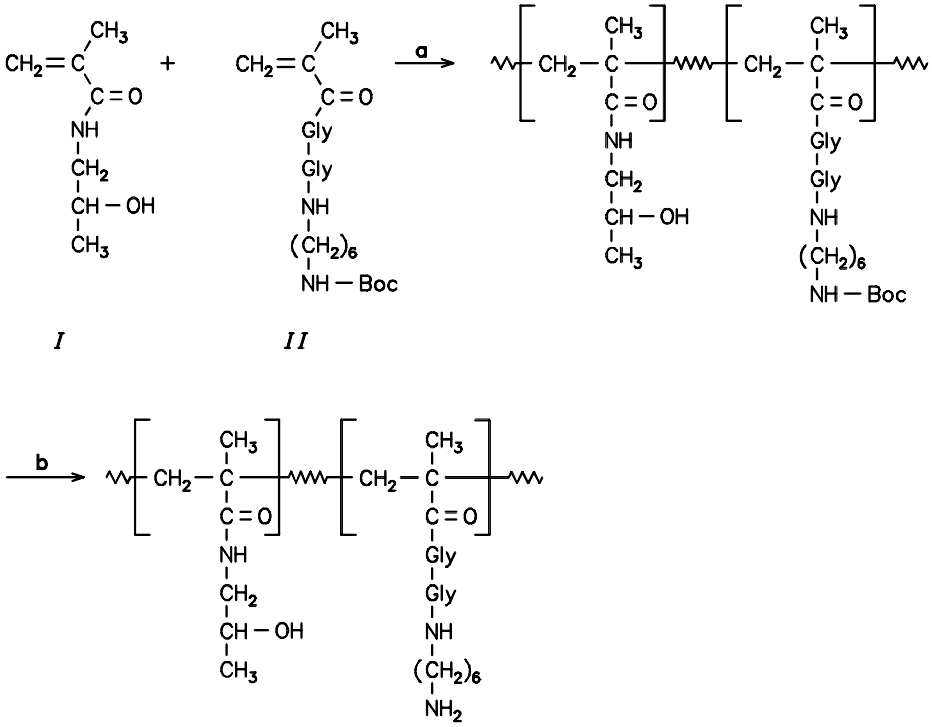
A solution of amide *I* (3 g, 21 mmol) and AIBN (0.38 g) in methanol (21.6 g) was polymerized in a sealed glass ampoule under nitrogen at 60 °C for 24 h. The product was precipitated by pouring the solution into a twentyfold excess of acetone, washed with acetone and diethyl ether, and dried. Yield of the homopolymer 2 g (67%).

Copolymer with a Schiff Base in Pendant Groups

Poly(*I*–*co*–*II*) was prepared by copolymerization of *I* (3 g, 21 mmol) with *II* (1.47 g, 3.7 mmol) induced by AIBN (0.56 g) in methanol (32.2 g) in the same way as the homopolymer; yield 2.5 g (56%). To remove the terminal *tert*-butyloxycarbonyl groups of type *II* monomeric units, the product was dissolved in methanol (10 ml), mixed with methanolic HCl (20%, 20 ml), and stirred at 20 °C for 1 h. The copolymer was precipitated with acetone and dried, dissolved in water, and neutralized with $NaHCO_3$ (pH ca 8.5). The solution was purified by dialysis, and the copolymer with free NH_2 end groups was isolated by lyophilization. The amino end groups were transformed into Schiff bases by reaction with glycolaldehyde (a sixfold molar excess with respect to NH_2) in a phosphate buffer at pH 7 (37 °C, 48 h).

RESULTS AND DISCUSSION

The synthetic route to the unmodified copolymer is shown in Scheme 1. The composition and molecular-weight characteristics of the copolymer and reference homopolymer are summarized in Table I. The reaction of the two polymers with glycolaldehyde (GA) was monitored by electronic absorption spectroscopy as well as by excitation and emission fluorescence spectroscopy. After the addition of GA, the copolymer system exhibits broad absorption bands at 255 and 310 nm, whereas no appreciable changes



I *II*

unmodified copolymer

a AIBN, MeOH, 60 °C; b HCl, MeOH
Boc – *tert*-butyloxycarbonyl

SCHEME 1

occur in the spectrum of the system containing the reference homopolymer (Figs 1*a* and 1*b*). Both of the observed absorption bands have their counterparts in the excitation fluorescence spectrum (Fig. 2, curve *3*). The emission fluorescence spectrum of the copolymer system consists of a very broad band with the maximum at 415 nm (Fig. 3). Figures 2 and 3 demonstrate that the corresponding bands are virtually absent from the fluorescence spectra of both GA and the homopolymer reacted with GA. The observed spectral changes are in a good agreement with those reported in the literature for the nonenzymatic glycosylation of proteins[8,12] which are ascribed to the formation of Schiff bases (SB). In fact, the band at 415 nm is known to be characteristic of fluorescence emission spectra of various Schiff bases (ref.[8]).

TABLE I

Characteristics of the polymers studied: x_{NH_2} is the mole fraction of monomeric units bearing amino groups, as determined by the ninhydrine method; $\overline{M}_w$ is the weight-average and $\overline{M}_n$ the number-average molecular weight of the polymer; $I_n = \overline{M}_w/\overline{M}_n$ is the index of molecular non-uniformity

Polymer	x_{NH_2}, %	$\overline{M}_w$	$\overline{M}_n$	I_n
Homopolymer	0	19 900	16 100	1.23
Copolymer	17.6	25 000	20 000	1.22

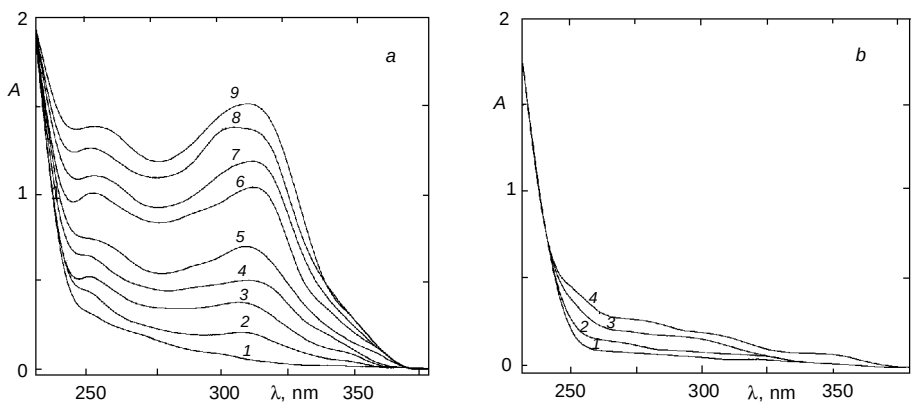
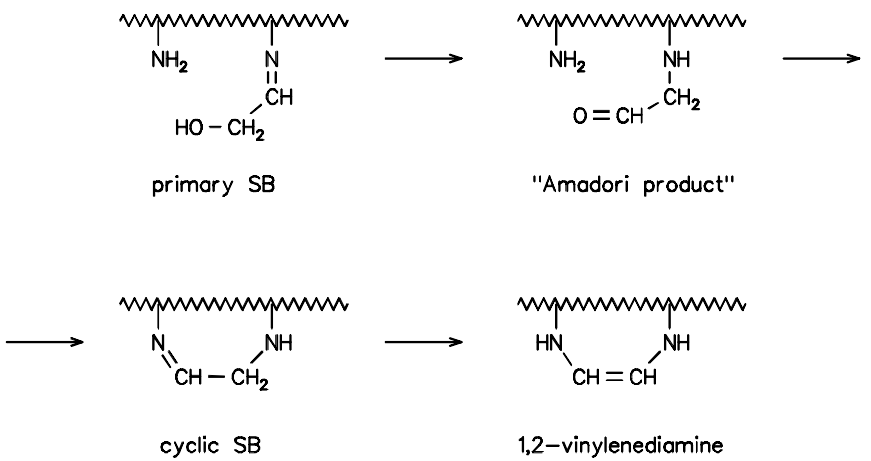


FIG. 1

Electronic absorption spectra of the unmodified copolymer (*a*) and homopolymer (*b*) (concentration of monomeric units 0.01 mol dm^{-3} in both cases), reacted with GA (0.01 mol dm^{-3}) in phosphate buffer pH 7 at 37 °C. Reaction time (h) in *a*: *1* 0, *2* 4, *3* 6, *4* 8, *5* 10, *6* 17, *7* 25, *8* 40, *9* 48; in *b*: *1* 0, *2* 5, *3* 25, *4* 48. The spectra were recorded at 20 °C

The kinetics of SB formation in phosphate buffers at pH 6, 7 and 8, respectively, is shown in Fig. 4. Both the rate of formation and the yield of SB are highly sensitive to pH of the reaction medium. As to the sigmoid shape of the kinetic curves, this is consistent with that found earlier by Marisa et al.[8] for the reactions of proteins with GA. The explanation suggested by those authors is based on the assumption that the absorbing species are formed in two consecutive steps at least: (i) formation of the primary SB, and (ii) subsequent reaction of the β-hydroxyl of the primary SB with another amino group giving the 1,2-vinylenediamine functional group. However, recent study accomplished in our laboratory[13] indicated that the primary SB is rearranged to the "Amadori product" (β-aminoaldehyde). In the next step, the condensation of the new carbonyl group with another terminal amino group resulting in the formation of the cyclic SB seems to be a more probable reaction pathway of the transformation studied (see Scheme 2).



SCHEME 2

Regardless of the mechanism, the condensation of the high-molecular-weight copolymer might potentially also proceed as an intermolecular reaction leading to crosslinking. This possibility was examined by viscometric measurements but the result was negative. This can be attributed to the low concentration of the copolymer in the reaction mixture preventing the primary SB from interaction with the amino groups of other macromolecules.

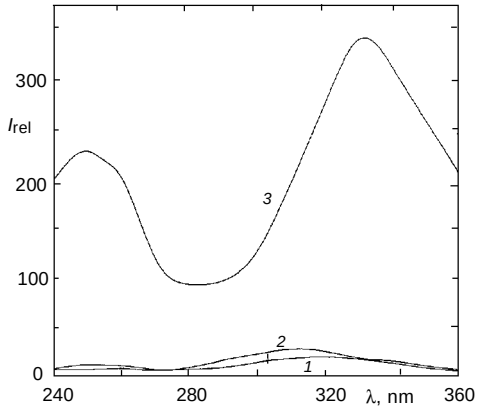


FIG. 2

Excitation fluorescence spectra of GA (0.01 mol dm^{-3}) (*1*), the homopolymer (*2*) and the copolymer (*3*) (concentration of monomeric units 0.01 mol dm^{-3} in both cases) after reaction with GA (0.01 mol dm^{-3}) in phosphate buffer pH 7 at 37 °C for 48 h. The spectra were recorded at 20 °C, λ_{emis} = 415 nm

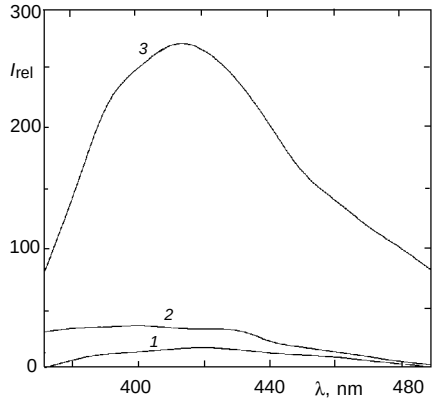


FIG. 3

Emission fluorescence spectra of GA (*1*) (0.01 mol dm^{-3}), the homopolymer (*2*) and the copolymer (*3*) (concentration of monomeric units 0.01 mol dm^{-3} in both cases) after reaction with GA (0.01 mol dm^{-3}) in phosphate buffer pH 7 at 37 °C for 48 h. The spectra were recorded at 20 °C, λ_{excit} = 330 nm

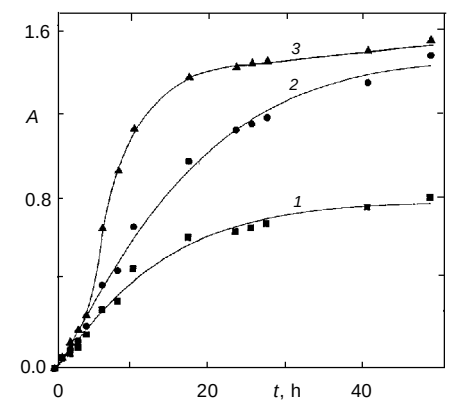


FIG. 4

Time dependence of absorbance of the reaction mixture at 310 nm during reaction of the copolymer (concentration of monomeric units 0.01 mol dm^{-3}) with GA (0.01 mol dm^{-3}) in phosphate buffer at pH: *1* 6, *2* 7, *3* 8, at 37 °C

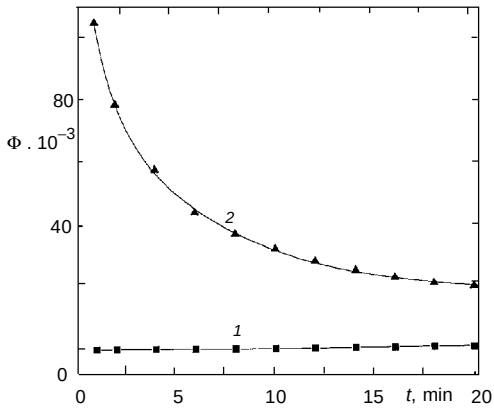
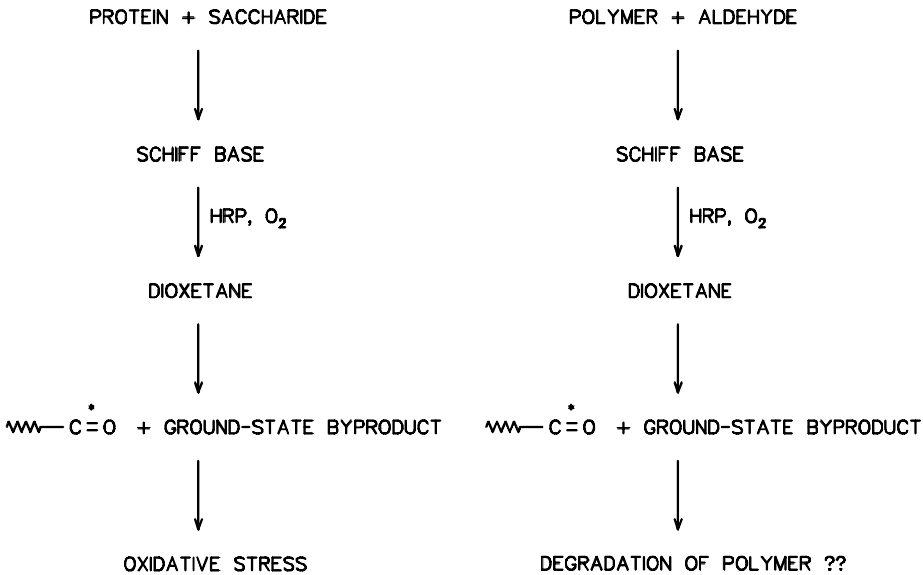


FIG. 5

Time dependence of chemiluminescence intensity Φ (counts per minute) of the homopolymer (*1*) and copolymer (*2*) solution (concentration of monomeric units 0.01 mol dm^{-3}) treated with GA (0.01 mol dm^{-3}) at pH 7 and temperature 37 °C for 48 h, after the addition of HRP (t = 0; resulting concentration 1 μmol dm^{-3})

The generation of electronically excited states during the interaction of the modified copolymer with dissolved atmospheric oxygen in the presence of HRP was monitored in two ways: (i) by direct chemiluminescence measurement and (ii) by the indirect method based on transfer of the excitation energy to bilirubin, followed by a fast decomposition of this "indicator" to green products[14]. The results of the direct measure-



SCHEME 3

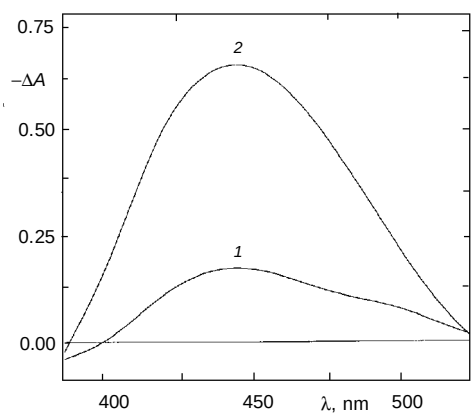


FIG. 6
Differential absorption spectra of the homopolymer (*1*) and copolymer (*2*) solution (concentration of monomeric units 0.01 mol dm^{-3}) treated with GA (0.01 mol dm^{-3}, pH 7, 37 °C, 48 h) and subsequently mixed with bilirubin (resulting concentration 50 μmol dm^{-3}). The spectra were recorded immediately after the addition of HRP (t = 0; resulting concentration 1 μmol dm^{-3}) into the measuring cell

ments (Fig. 5) demonstrate that chemiluminescent radiation is emitted during the enzymatically catalyzed oxidation of the modified copolymer whereas no signal exceeding the natural background appears in the case of the homopolymer treated in the same way as the copolymer. The results of the indirect detection method (Fig. 6) are in a qualitative agreement with those obtained by the direct measurement. The indirect detection of electronically excited states was carried out by the differential technique in order to compensate for the photochemical decomposition of bilirubin by the primary light beam. The reference solution only differed from the sample solution by the absence of HRP.

In summary, the water-soluble copolymer with terminal amino groups at the short side chains was synthesized and characterized. The transformation of the copolymer amino groups into SB was found to proceed in a way similar to the case of proteins giving the product (SB) with similar spectroscopic characteristics. The modified copolymer was demonstrated to emit radiation during its oxidation catalyzed by HRP. Owing to their relatively good availability and to the possibility of controlling the free amino group content, copolymers of this type can be used with advantage as model compounds for studying the mechanism of enzymatically catalyzed generation of electronically excited states of proteins. In addition, the knowledge gained through these studies might be utilized in designing a new type of biodegradable polymers as suggested in Scheme 3.

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