JOURNAL OF MATERIALS SCIENCE 30 (1995) 729–733

Photocatalytic degradation of heparin over titanium dioxide

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The photochemical degradation of the homogeneous oxygen-saturated buffered aqueous solutions of sodium heparin salt initiated by the irradiation with medium pressure mercury lamp was compared with the heterogeneous photocatalytic heparin degradation sensitized by titanium dioxide. The obtained results confirmed that UV irradiation of heparin homogeneous aqueous solutions caused negligible changes in the polysaccharidic structure only. The irradiation of heparin in oxygen-saturated physiological saline photosensitized by the TiO_2 caused significant changes in the heparin macromolecule. The study of photocatalytic heparin degradation in buffered oxygen-saturated TiO_2 suspensions (pH = 5–9) ascertained a significant influence of pH on the degradation of heparin. The polymeric chain is predominantly cleaved in the acidic and neutral medium.

1. Introduction

Heparin is a polydisperse, highly sulphated, linear polysaccharide. Heparin chains consist of the repetition of 10–50 disaccharide units made up of a uronic acid and a 2-amino-2-deoxy-D-glucose unit [1]. The repeating disaccharide unit **1** [→4)-α-L-idopyranosyluronic acid 2-sulphate-(1→4)-2-deoxy-2-sulphoamino-α-D-glucopyranosyl 6-sulphate-(1→] shown in Fig. 1 accounts for at least 85% of heparin from beef lung and about 75% of those from intestinal mucosa [2]. However, other units (Fig. 1) such as, nonsulphated α-L-iduronic acid (**2**), β-D-glucuronic acid (**3**), nonsulphated (**5**) and sulphated 2-acetamido-2-deoxy-α-D-glucose (**4**), and 2-deoxy-2-sulphoamino-α-D-glucose (**8**) and its 3-sulphate (**7**) and 3,6-disulphate (**6**) derivatives also occur to different extents in place of the respective units in **1** depending on the origin of the heparin [1].

Heparin and heparin fragments (low molecular weight heparins, LMWH) are currently used in medicine for the prevention and the treatment of thrombosis. The antithrombotic activity is related to the anticoagulant properties of the polysaccharide. Heparin also displays many other pharmacological activities [1, 3, 4]. LMWH are prepared by controlled, partial, chemical or enzymatic depolymerizations of commercial heparins. These LMWH are polydisperse, with average molecular weight around 5000, considerably variable in chemical structure, anticoagulant activity, and experimental antithrombotic effects. The main characteristics of some presently available LMWH were published by Coccheri [5].

Balazs *et al.* studied the effect of UV radiation on heparin aqueous solution [6]. Nagasawa *et al.* investigated in detail the chemical changes in heparin macromolecules involved in the oxidative–reductive depolymerization of heparin [7]. Their results indicate that heparin undergoes a preferential destruction of the D-glucuronic acid residues, which are one of the essential constituents of the antithrombin-binding saccharide sequence, and this change in the structure causes a marked decrease in the affinity for the proteins and also in the related anticoagulant activity [7].

The irradiation of the oxygen-saturated aqueous suspensions of titanium dioxide leads to the formation of active oxygen radical intermediates [8] as is shown in the following scheme:

$$TiO_2 + h\nu \xrightarrow{h\nu > E_{bg}} TiO_2(h^+ + e^-) \quad (1)$$

$$O_2 + e^- \rightarrow O_2^- \quad (2)$$

$$OH^- + h^+ \rightarrow {}^{\cdot}OH \quad (3)$$

$${}^{\cdot}OH + \text{substrate} \rightarrow \text{degradation products} \quad (4)$$

where h^+ is a photogenerated hole, e^- is a photogenerated electron and E_{bg} is the band gap energy of titanium dioxide.

This work is an attempt to apply the photocatalytic method to reduce the molecular weight of commercial heparin in the heterogeneous photochemical degradation process sensitized by titanium dioxide.

2. Experimental procedure

Titanium dioxide P25 (Degussa, FRG) was used in all photocatalytic experiments. Degussa P25 titanium dioxide is predominantly anatase (80% anatase, 20% rutile), with surface area $50 \pm 5\ m^2\ g^{-1}$ and with energy band gap of 3.18 eV [9]. The sodium salt of

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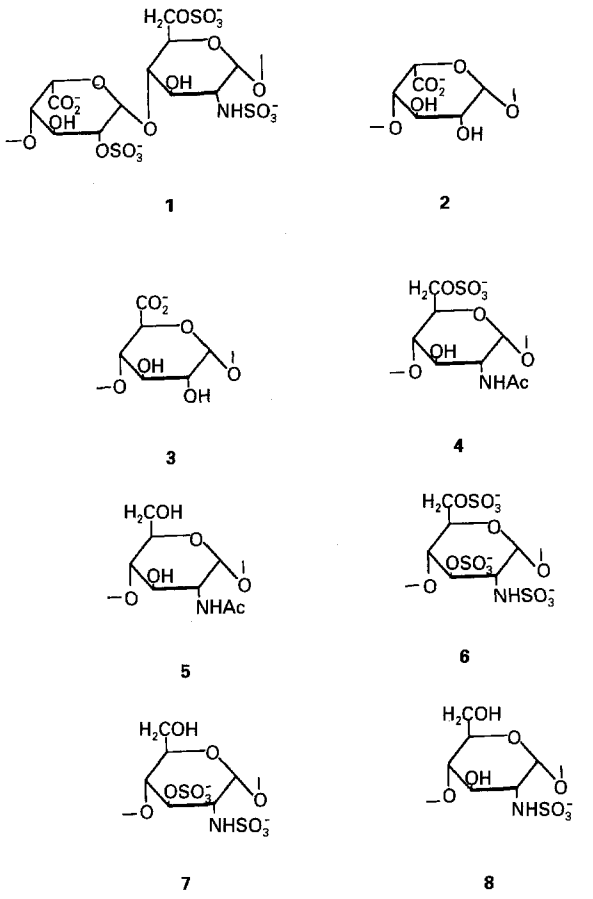

Figure 1 Fundamental disaccharide unit of heparin (**1**) and other saccharidic structures present in heparin polymer chain (**2–8**).

beef lung heparin ($M_w = 6100$) was purchased from Léčiva (Czech Republic) and was used without further purification. The solutions and suspensions of heparin were prepared using physiological saline or phosphate buffers (in redistilled water); the heparin concentration was 0.5 wt %; titanium dioxide concentration was 0.8 g dm^{-3}. All chemicals used were of analytical grade purity.

The photoreactions were carried out at a temperature of 25 °C in the photochemical quartz immersion-well (Applied Photophysics, UK). As the radiation source, a 125 W medium pressure mercury lamp was used (Applied Photophysics, UK). During irradiation the reaction mixtures were continuously bubbled with carbon dioxide free oxygen (flow rate 500 ml min^{-1}) and were stirred by magnetic stirrer. When necessary the samples removed from the reactor were immediately centrifuged for 20 min at 5000 *g* on the MLW T62.2 centrifuge (FRG) and were filtered on Millipore microfilters (0.22 μm). The pH values of the samples were measured on a Radelkis OP-211/1 (Hungary) pH-meter; a combined glass electrode was used.

The UV-visible spectra were monitored on a Philips PU 8800 spectrophotometer in a cell path of 1 or 0.2 cm length. Infrared spectra were measured on a Perkin–Elmer 599 IR spectrophotometer; the KBr technique was used. For the IR spectra run, heparin precipitated from aqueous solutions using methanol was filtered and carefully dried at 60 °C.

The distribution of heparin macromolecules was monitored by gel chromatography using Frequency Power Liquid Chromatograph (FPLC) system (Pharmacia, Sweden) equipped with chromatographic control unit LCC-500 PLUS for monitoring UV-M. The column Superose 6 (10 × 300 mm) and the detector wavelength $\lambda = 214$ nm were used in all FPLC analysis. A mixture of 0.15 M aqueous NaCl and 0.06 M Na_2HPO_4 (1:1) was applied as a mobile phase [10].

Carbon dioxide evolved during photoreactions was determined conductometrically by means of a Radelkis OK 102/1 (Hungary) conductometer as described elsewhere [11]. Electron paramagnetic resonance (EPR) spectra were measured using a Bruker 200E spectrometer coupled to an Aspect 2000 computer (FRG). The spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, Sigma), was freshly distilled before use (75 °C and 0.5 Torr) and stored at − 25 °C under argon. The samples for EPR spectra monitoring were placed in a flat quartz cell and irradiated directly in the TM 102 cylindrical cavity. As an irradiation source 250 W medium pressure lamp (Applied Photophysics, UK) was used. Viscometric measurements were performed on capillary Ubbelohde viscometers. The viscosity was determined at 25 °C and the values of intrinsic viscosity [η] were calculated. The concentration of aminosaccharides was determined after hydrolysis of heparin samples in H_2SO_4 by the Morgan spectrophotometric method [12]. The concentration of ammonium ions were measured using Nessler reagent spectrophotometrically [13]. The concentration of sulphate ions was determined nephelometrically as $BaSO_4$ [13]. The concentration of reducing saccharides was investigated after hydrolysis of heparin samples by the chelatometric method [14].

3. Results and discussion

The UV irradiation of homogeneous solutions of macromolecules causes the excitation of polymer electronic levels which results in various chemical changes. The possible mechanisms include polymer bond cleavage and free radical formation, photocyclization, intramolecular rearrangement and fragmentation. It was assumed that the polymer chain degradation is initiated by photogenerated reactive oxygen radicals [15]. The oxidation of organic compounds in the aqueous titanium dioxide suspensions proceed mainly via photogenerated hydroxyl radicals [8, 16].

The formation of hydroxyl radicals during the irradiation of heparin solutions or suspensions was monitored by spin trap technique using DMPO. The ·DMPO-OH adduct, characterized by a quartet (1:2:2:1) spectrum with hydrogen and nitrogen splitting constants 1 mili Tesla = 10 Gauss and *g*-factor = 2.0058 (see Fig. 2) was observed in the irradiated homogeneous oxygen-saturated heparin solutions (pH = 7) only in negligible concentration. The irradiation of oxygen-saturated titanium dioxide suspensions of heparin (pH = 7), however, immediately caused the significant formation of ·DMPO–OH adduct as shown in Fig. 2.

Any changes in the UV spectra of heparin were

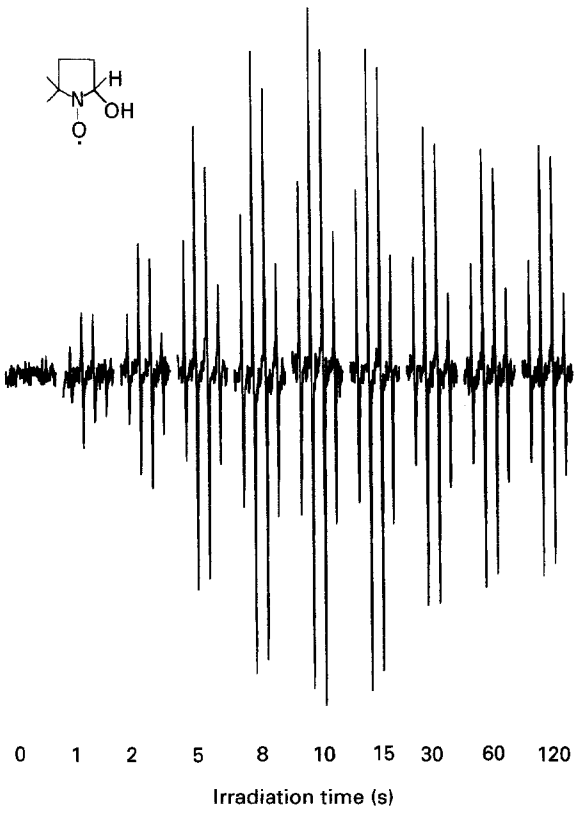

Figure 2 EPR spectra of DMPO–OH adduct formed during discontinued irradiation of buffered oxygen-saturated heparin TiO_2 suspension (pH = 7).

range 4–9 after 2 h of irradiation. Fig. 3a, b illustrate the UV spectra of heparin in physiological saline after addition of distilled water, 0.1 M HCl and 0.1 M NaOH. The modification of pH values caused only unimportant changes in UV spectra of non-irradiated heparin (Fig. 3a). The significant absorption band at $\lambda = 294$ nm is formed after NaOH addition in the heparin sample irradiated for 2 h in TiO_2 suspension (Fig. 3b). This band was attributed to the saccharidic ionizable chromophoric group [17].

The IR spectra of original and heparin irradiated in TiO_2 suspensions for 2 h were measured using the KBr technique (Fig. 4). Grant *et al.* [18] analysed IR spectra of natural and chemically modified heparins and they suggested that the region of 1500–1100 cm^{-1} is very important for the identification of heparin functional groups. The IR spectrum of irradiated heparin could be after comparison with published spectra [18] probably attributed to the *N*-desulphonated heparin.

The irradiation of heparin in physiological saline photosensitized by TiO_2 caused significant changes in macromolecule after 3 h of irradiation; i.e. 80% increase of reducing saccharide concentration; the concentration of heparin amino and sulphate groups decrease, the 44% increase of NH_4^+ concentration was determined in solution; the molecular weight calculated from the Mark–Houwing equation [19] decreases from 6100 to 3500; carbon dioxide is evolved during irradiation (Fig. 5). The carbon dioxide originates from decarboxylated heparin glucuronic units,

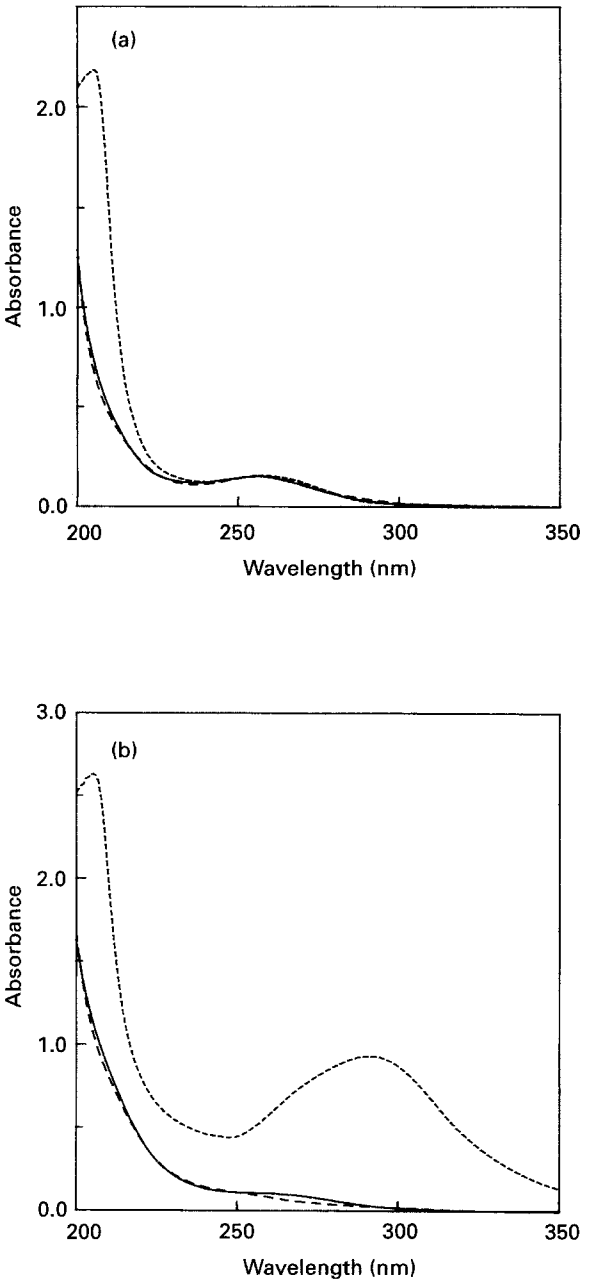

Figure 3 UV spectra of heparin in physiological saline (0.5 wt%) after addition of distilled water, 0.1 M HCl and 0.1 M NaOH. (1 ml of heparin solution was mixed with 1 ml of additive solution; cell length 0.2 cm). (a) non-irradiated heparin; (b) heparin irradiated for 2 h in oxygen-saturated TiO_2 suspension. ———, distilled water; – – –, 0.1 M HCl; ------, 0.1 M NaOH.

catalytic reaction on TiO_2. This assumption is confirmed by the important pH value drop from 6.3 to 3.7 during irradiation.

Table I shows the results of the elementary analysis for the original sample and heparin irradiated for 2 h in TiO_2 suspension prepared in physiological saline. The irradiation of heparin in homogeneous physiological saline under the experimental conditions, cause only unmeasurable changes.

The pH value of the photocatalytic TiO_2 systems is a very important parameter which significantly influence the polymer degradation process. Therefore further experiments with heparin were carried out in the

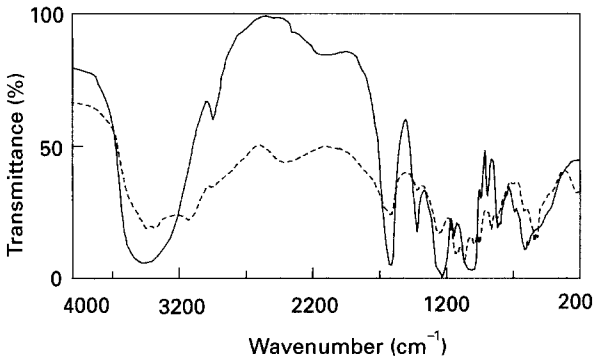

Figure 4 IR spectra of heparin sample (KBr technique). ——— original heparin sample; ----- heparin irradiated for 2 h in oxygen-saturated TiO_2 suspension.

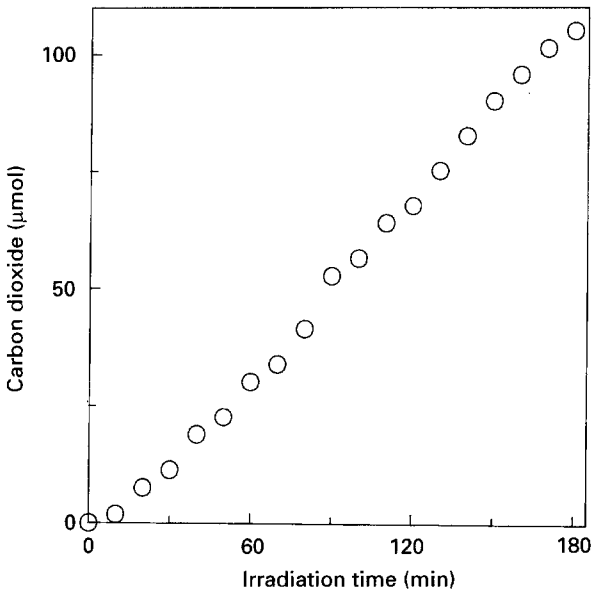

Figure 5 Carbon dioxide formation during photocatalytic decomposition of heparin in oxygen-saturated TiO_2 suspension prepared in physiological saline.

TABLE I The results of elementary analysis for original heparin sample and for samples irradiated in oxygen-saturated TiO_2 suspensions prepared in physiological saline

	Element content (%)			
Exposure	Nitrogen	Carbon	Hydrogen	Sulphur
0	2.3	20.0	3.3	9.3
2	1.8	18.5	3.1	7.6
3	0.9	8.88	1.2	2.8

buffered (pH = 5, 6, 7, 8, 9) systems and the effect of pH on heparin degradation was investigated.

The results of the intrinsic viscosity determination for the natural and irradiated buffered TiO_2 suspensions (1 h) of heparin are shown in Table II. These results confirm the dependence of the heparin polymer chain degradation on the pH values of the medium. Heparin is a highly negatively charged polysaccharide, and the orientation as well as arrangement of its chain is strongly pH dependent. Thus arrangement of the polymer could influence the centre of hydroxyl radical

TABLE II The values of intrinsic viscosity (25 °C) of heparin in the pH region 5–9 obtained for non-irradiated and for 1 h-irradiated TiO_2 systems

	$[\eta]$ (dl g^{-1})	
pH	Original heparin	Irradiated heparin
5	0.44	0.31
6	0.43	0.16
7	0.49	0.23
8	0.32	0.26
9	0.31	0.20

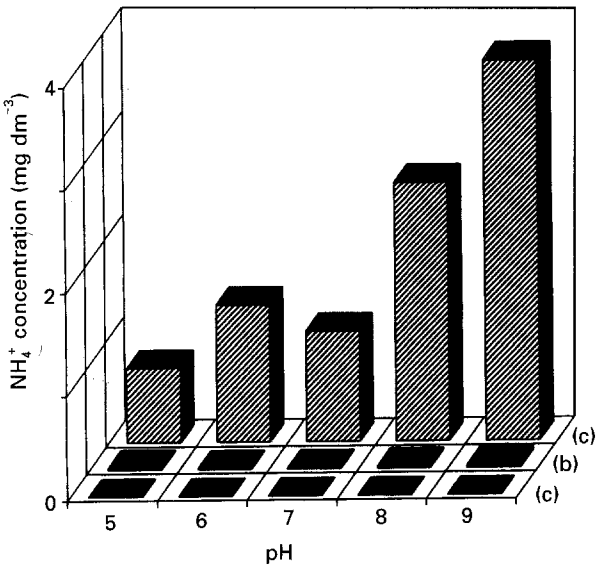

Figure 6 Concentration of NH_4^+ ions determined in non-irradiated and after 1 h irradiation in buffered heparin systems with various pH values. (a) non-irradiated; (b) homogeneous solutions; (c) heterogeneous TiO_2 suspensions of heparin.

attack in the heparin photocatalytic reaction on TiO_2. The gel chromatography data confirm the proposed sensitivity of heparin photocatalytic degradation on pH values. The extension of polymer molecular weight distribution was significant especially in acidic solutions.

Fig. 6 illustrates the dependence of the NH_4^+ concentration on the pH values in non-irradiated (a), in homogeneous (b), and in heterogeneous (c) systems after 1 h of irradiation. The concentration of NH_4^+ in the irradiated homogeneous heparin solutions is negligible. The increase in medium alkalinity leads to the growth of the photocatalytic cleavage of the amino group from heparin.

Fig. 7 shows the dependence of the SO_4^{2-} concentration on pH values in non-irradiated (a), in homogeneous (b), and in heterogeneous (c) systems after 1 h of irradiation. The concentration of the measured SO_4^{2-} decreases with the increase in medium pH values for the irradiated systems.

Under our experimental conditions the photochemical degradation of heparin in the homogeneous aqueous solutions is negligible. The presence of titanium dioxide significantly increases the effectiveness of the photochemical polymer degradation. The

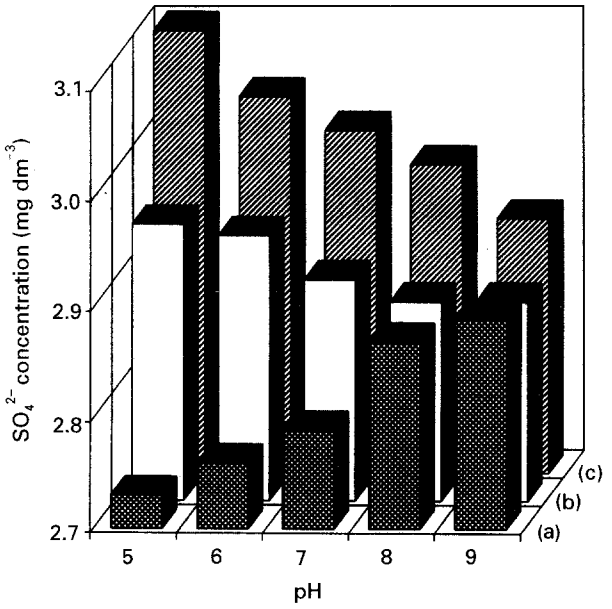

Figure 7 Concentration of SO_4^{2-} ions determined in non-irradiated and after 1 h irradiation in buffered heparin systems with various pH values. (a) non-irradiated; (b) homogeneous solutions; (c) heterogeneous TiO_2 suspensions of heparin.

photocatalytic degradation of heparin sensitized by TiO_2 is a very complex process (parallel polymer chain cleavage and functional group decomposition) and the preparation of LMWH depends on the exact reaction condition definition and stabilization. The studies of the dependence of the biological activities of photocatalytically prepared heparins on the polymer structure changes is the main aim of our further investigations.

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Received 22 October 1993
and accepted 27 July 1994