SYNTHETIC HEPARINOIDS LABELLED WITH ^{125}I AND ^{35}S

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KEYWORDS

Polyelectrolyte

Heparinoid

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SUMMARY

The labelling of a water-soluble synthetic polyelectrolyte, having anticoagulant activity, has been studied. The polyelectrolyte is derived from cis-1,4-polyisoprene and contains N-sulfate and carboxylate groups. [^{125}I]-Iodination of the polyelectrolyte, using the Chloramine-T method and an electrolytic method, resulted in a [^{125}I]-labelled polyelectrolyte from which release of the label occurred. Resulfation of a partially desulfated polyelectrolyte with a [^{35}S]-sulfur trioxide trimethylamine complex resulted in a [^{35}S]-labelled polyelectrolyte which showed no release of the label.

INTRODUCTION

In previous publications (1-7) we reported on the preparation of a water-soluble polyelectrolyte having anticoagulant activity (2-4,6,7). The polyelectrolyte is derived from cis-1,4-polyisoprene and contains the following structural units, apart from unreacted

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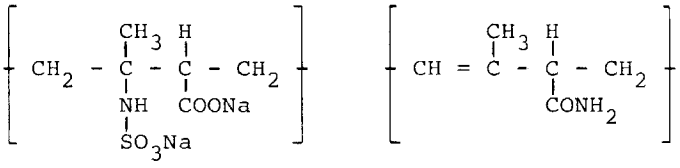
isoprene units (5):



There is a resemblance to heparin because of the presence of N-sulfate and carboxylate groups in the structure. Moreover the polyelectrolyte acts, just like heparin, as an antithrombin (2) and the anticoagulant activity of heparin and the polyelectrolyte are related to the molecular weight and the N-sulfate content (4, 6, 7).

Heparin, as well as the polyelectrolyte can be linked ionically to polymer surfaces by means of an adsorptive coupling agent (e.g. tridodecylmethylammonium chloride (TDMAC)) (6, 8).

Polymer surfaces, coated with the TDMA-polyelectrolyte complex showed reduced platelet adhesion, when exposed to freshly drawn human blood (9, 10). However, like the TDMA-heparin coating (11), these coatings were not stable in plasma and phosphate buffered saline, and loss of polyelectrolyte from the surface occurred (6).

The grafting of low molecular weight polyelectrolyte onto silicone rubber surfaces and the crosslinking of high molecular weight polyelectrolyte in the pores and near the surface of a polystyrene resin, using ^{60}Co gamma radiation, has been studied recently (12). The presence of polyelectrolyte on the surface could be shown with the dye Azur A. In vitro studies with human blood indicated that the coated surfaces showed less platelet adhesion than the uncoated ones. For optimization of the grafting conditions and for the study of the stability of the polyelectrolyte graft layer, it is necessary to know the amount of polyelectrolyte on the surface. The polyelectrolyte graft layer could not be detected by weighing, Attenuated Total Reflection spectroscopy (ATR) or by Energy Dispersive Analysis of X-rays (EDAX). Measuring the activity

of a radioactive label is a more sensitive detection method and therefore the presence of grafted polyelectrolyte might be studied using the compound in a radioactively labelled form.

In this publication we report on the preparation of [^{125}I]- and [^{35}S]-labelled polyelectrolytes. Labelling of the polyelectrolyte with ^{125}I was based upon the assumption that iodine can add to the carbon-carbon double bonds in the polyelectrolyte molecule. A solution of iodine in water is decolourized by the addition of polyelectrolyte, when a small excess of double bonds are available. Froehling (13) and Robinson and Lee (14) already reported on the labelling of unsaturated compounds by the addition of ^{125}I to the double bonds.

Labelling of the polyelectrolyte with ^{35}S is based upon a method described by Lloyd et al. (15) for the labelling of heparin. N-Sulfate groups, which are present in heparin and the polyelectrolyte, are not stable in an acidic medium and rupture of N-S bonds takes place (4, 6, 7). After partial desulfation, resulfation can be carried out with a [^{35}S]-sulfur trioxide trimethylamine complex.

EXPERIMENTAL

Materials

Radiochemicals

[^{125}I]-Sodium iodide (carrier free, in sodium hydroxide solution, pH 7-11, 100 mCi/ml) and [^{35}S]-sulfur trioxide trimethylamine complex ($^{35}SO_3N(CH_3)_3$) (21 mCi/mmol), solid) were purchased from Amersham International plc, Amersham (UK).

Polyelectrolytes

Polyelectrolytes PLE-H and PLE-L were synthesized as has been described (1, 5).

The starting material for the synthesis of PLE-H was cis-1,4-polyisoprene (Cariflex IR 307, Shell), with a molecular weight of

$\bar{M}_n$ = 240.000, $\bar{M}_w/\bar{M}_n$ = 5.3. (as determined by Gel Permeation Chromatography (GPC)). For the synthesis of PLE-L cis-1,4-polyisoprene with a low molecular weight ($\bar{M}_n$ = 6.700, $\bar{M}_w/\bar{M}_n$ = 1.1) was used. The polyelectrolytes were purified by dialysis against distilled water for three days, followed by freeze-drying of the solutions. For the dialysis of PLE-L solutions benzoylated dialysis tubing (Sigma D 7884) was used and for the dialysis of solutions of PLE-H normal cellulose tubing (Cenco).

Partially desulfated polyelectrolytes

For the preparation of partially desulfated polyelectrolytes (PLE-H_d and PLE-L_d) portions of polyelectrolyte were brought into 0.1 M HCl at 20°C for 50 hours. Subsequently sodium hydroxide was added up to pH ≈ 7, followed by dialysis of the solutions against distilled water for three days. The reaction products were isolated by freeze-drying of the solutions.

[^{125}I]-labelled polyelectrolytes

The [^{125}I]-labelled polyelectrolytes were obtained by iodination of the polyelectrolyte with ^{125}I, using a. the Chloramine-T method (16, 17), and b, an electrolytic method (18, 19).

[^{35}S]-labelled polyelectrolytes

The [^{35}S]-labelled polyelectrolytes were prepared by resulfation of the amino groups of partially desulfated polyelectrolytes with a [^{35}S]-sulfur trioxide trimethylamine complex.

200 mg of partially desulfated polyelectrolyte was dissolved in 4 ml water and the solution was adjusted to pH = 9.5 by the addition of 1 M NaOH. Then the solution was heated to 55°C, followed by the addition of 20 mg of sodium bicarbonate. Under vigorous stirring 1-2 mg of [^{35}S]-sulfur trioxide trimethylamine complex (and sometimes 18-19 mg of unlabelled complex) were added (molar ratio: total sulfur trioxide/amino groups ≤ 1), while the pH was kept constant at 9.5 by the addition of 1 M NaOH. The alkaline solution was kept at 55°C for 24 hours. After cooling to 20°C 100 mg of sodium

chloride was added to the solution. The polyelectrolyte was separated from low molecular weight compounds by dialysis or by gel filtration.

Methods

Radioactivity measurements

For the measurements of the radioactivity of the [^{125}I]-labelled compounds, two milliliter samples were counted in a 3"x3" NaI (Tl) Well type scintillation detector, coupled to a Tracor Northern Econ II multichannel analyser.

The samples, containing [^{35}S]-labelled products, were counted in a Packard Tricarb Model 2650 Liquid scintillation counter, using 10 ml of "Lumagel" scintillation medium and 1 ml of liquid sample for each measurement.

Separation of polyelectrolytes from low molecular weight compounds after the labelling procedure

a. Dialysis

The polyelectrolytes could be separated from low molecular weight products by dialysis. For this purpose benzoylated dialysis tubing (Sigma D 7884) was used for the dialysis of solutions of PLE-L and Visking dialysis tubing (Servo) was used for the dialysis of solutions of PLE-H. Before dialysis the tubing was treated with an unlabelled polyelectrolyte solution (c = 10 mg/ml) to minimize the adsorption of labelled polyelectrolyte to the membrane.

The reaction mixtures were dialyzed against 250 ml of distilled water. After certain intervals samples were taken from the dialysate for radioactivity measurements, immediately followed by changing the dialysate. After dialysis the purified solutions were transferred to a volumetric flask (10-100 ml) and filled up with water. Also from this solution a sample was taken for radioactivity measurements. The polyelectrolytes were isolated by freeze-drying of the solutions.

b. Gel filtration

Sephadex G 25, previously swollen in 0.05 M sodium phosphate buffer (pH = 7.5) or 0.16 M sodium chloride solution, was used to prepare a column (0.9 x 12 cm). To minimize the adsorption of labelled polyelectrolyte, the column was treated with unlabelled polyelectrolyte. Therefore 0.5 ml of a solution of 100 mg/ml polyelectrolyte in 0.05 M sodium phosphate buffer was passed through the column.

Samples of 20-200 µl were applied to the column, which was then eluted with 0.05 M sodium phosphate buffer or 0.16 M sodium chloride, at a flow rate of 25 ml/hr. The eluate was collected in 0.5 ml fractions and the radioactivity in all the fractions was measured. The recovery of radioactivity, applied to the column was 70-100%.

Thin layer chromatography

During and after the electrolytic iodination, the percentage of ^{125}I, bound to the polyelectrolyte was determined by thin layer chromatography (19).

The plates used were D.C. Alufolien, Cellulose F254, Merck no 5574/0025. Methanol was used as the solvent.

Thrombin times

For the determination of the anticoagulant activity thrombin times of citrated human plasma were measured, according to a modification of the method of Studer and Winterstein (20). The modification of this method is described in detail elsewhere (7).

RESULTS AND DISCUSSION

Iodination by the Chloramine-T method

In the case of proteins and polypeptides labelling is readily accomplished by iodination with ^{125}I. The most widely used method of iodination is the oxidation of $Na^{125}I$ by Chloramine-T in the presence of a protein. Oxidation of $Na^{125}I$ by Chloramine-T leads

to the formation of radioactive I^+ and I_2 which substitute in the phenolic rings of tyrosine of the protein (17). Chloramine-T can be used for iodination in substitution as well as in addition reactions (13).

Before iodination of the polyelectrolytes with ^{125}I, reactions were carried out with unlabelled reagents. The polyelectrolytes were isolated after dialysis and it was observed that the anticoagulant activity (thrombin time) had remained the same, indicating that the iodination reaction had no effect on the activity.

Iodination of the polyelectrolytes with ^{125}I was carried out varying the amount of polyelectrolyte and the oxidation time. As a control for the quality of the reagents two iodination reactions were carried out with albumin. After iodination the reaction mixtures were dialyzed and it was found that neither the concentration and molecular weight of the polyelectrolyte, nor the oxidation time was of real importance for the labelling.

After dialysis for more than 76 hours the radioactivity of the labelled albumin reaction mixture in the dialysis bag remained constant and a yield of about 50% was found for the albumin labelling procedure. For the polyelectrolytes, however, the radioactivity in the dialysis bag was still decreasing, even after dialysis for 165 hours. This means that ^{125}I was not firmly bound to the polyelectrolyte, giving rise to a permanent release of ^{125}I from the polyelectrolyte. In order to obtain more data about the stability of the [^{125}I]-labelled polyelectrolytes, reaction mixtures were analyzed by gel filtration. Similar results were obtained with PLE-L and with PLE-H.

Also these experiments showed that iodination of the polyelectrolytes, using the Chloramine-T method, resulted in [^{125}I]-labelled polyelectrolytes from which release of the label occurred.

Iodination by the electrolytic method

The polyelectrolytes were also labelled by electrolytic iodination. The percentage of radioactivity, bound to the polyelectrolyte and determined by thin layer chromatography, was 69.8% after 1.5, and 79.9% after 5 hours of electrolysis, respectively. By dialysis and thin layer chromatography it was shown that the percentage of radioactivity in the polyelectrolytes decreased with time. Also from these data it can be concluded that [^{125}I]-labelled polyelectrolytes were obtained to which ^{125}I was not stably bound, resulting in a release of ^{125}I from the polyelectrolytes.

Labelling with ^{35}S

Part of the N-S bonds in the N-sulfate groups were cleaved by reaction with HCl, leading to polyelectrolytes PLE-H_d and PLE-L_d, respectively. Before the partially desulfated polyelectrolytes were labelled by reaction with a [^{35}S]-sulfur trioxide trimethylamine complex, resulfation was studied with unlabelled complex (reaction conditions for the preparation of the polyelectrolytes PLE-H_{dr} and PLE-L_{dr} are shown in Table I).

TABLE I Analytical data of modified polyelectrolytes

PLE	C/N	N (mmole/g)	S (mmole/g)	N in N-sulfate / total N	N-desulfated (mmole/g)	N-S not broken %
PLE-H	8.13	3.84	2.61	0.68	-	-
PLE-H_d	8.34	4.20	2.14	0.51	0.71*	75.0°
PLE-H_{dr}	8.02	4.04	2.14	0.53	0.61	77.9
PLE-L	7.80	3.71	2.89	0.78	-	-
PLE-L_d	7.79	4.19	2.31	0.55	0.96	70.5
PLE-L_{dr}	7.68	4.04	2.31	0.57	0.85	73.1

* (0.68 - 0.51) x 4.20 ° (0.51/0.68) x 100%.

As calculated from elemental analysis the C/N ratios were about the same in the H-series as well as in the L-series, indicating that C-N bonds had not been broken during the reaction. It can also

be seen that in the partial desulfation of polyelectrolytes PLE-H and PLE-L about 25% and 29% of the N-S bonds were broken, respectively, leading to a decrease in the anticoagulant activity (Table II).

TABLE II Anticoagulant activity of the polyelectrolytes

Polyelectrolyte	Thrombin time (s)	Number of experiments (n)	S.D. (s)
PLE-H	18.9	8	0.8
PLE-H_d	14.0	8	0.2
PLE-H_{dr}	16.5	8	0.4
PLE-L	18.1	8	0.7
PLE-L_d	15.5	8	0.6
PLE-L_{dr}	16.1	8	0.4

Resulfation of the NH_2 groups of the polyelectrolytes PLE-H_d and PLE-L_d, using about an equimolar amount of sulfur trioxide trimethylamine complex, occurred to a small extent only (Table I). Partial resulfation resulted in a slight increase in the anticoagulant activity (Table II), as was expected (4,6,7).

The differences between the values, mentioned in Table II, are statistically significant ($p < 0.05$).

In Table III the reaction conditions of the resulfation experiments are given. One (blank) experiment was carried out without polyelectrolyte.

After resulfation the polyelectrolytes were separated from low molecular weight compounds by dialysis (Figure 1) or by gel filtration (Figure 2).

Dialysis of the reaction mixture without polyelectrolyte indicates, that removal of the low molecular weight compounds is quite effective. Resulfation of the partially desulfated polyelectrolytes PLE-H_d and PLE-L_d with [^{35}S]-sulfur trioxide trimethylamine complex resulted in the formation of [^{35}S]-labelled polyelectrolytes with stably incorporated label (Figure 1). The yield of the labelling

TABLE III Reaction conditions for the resulfation experiments

PLE	Amount (mg)	Amount of labelled + unlabelled complex (mg)	Radioactivity in dialysis bag after 140 hrs of dialysis (% of the initial value)
-	0	1°	1.4
PLE-H_d	200	20*	- **
	200	1°	13.5
	200	20	14.7
PLE-L_d	200	20*	- °°
	200	1°	8.7
	200	20	9.9

* only unlabelled complex
° only labelled complex
** preparation of PLE-H_{dr}
°° preparation of PLE-L_{dr}

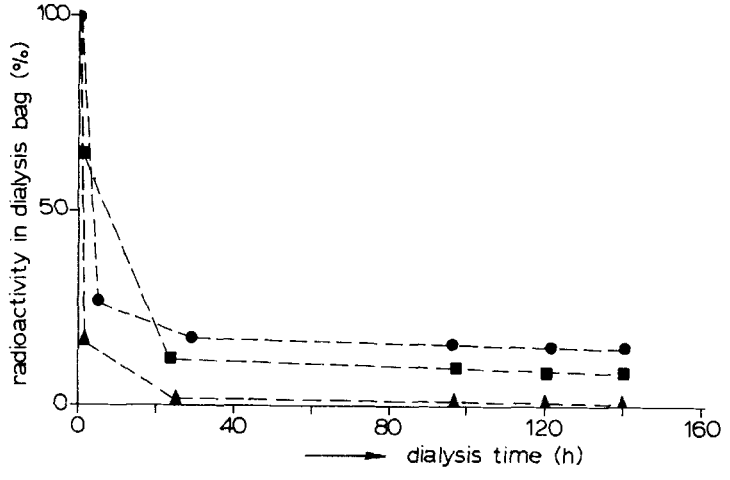
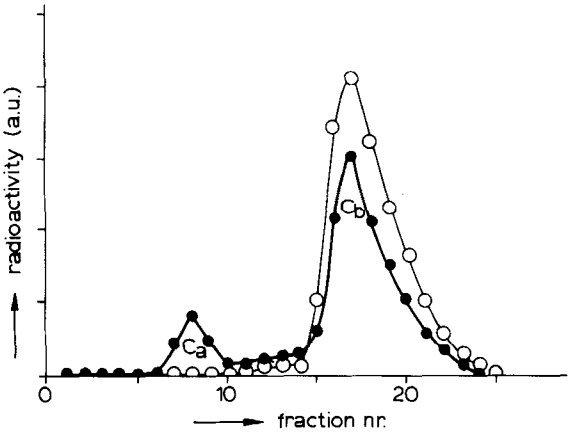


FIGURE 1

Radioactivity in dialysis bag as a function of time for the dialysis of a mixture of [^{35}S]-labelled polyelectrolyte and [^{35}S]-containing low molecular weight compounds.

- ● *200 mg PLE-H_d + about 1 mg of [^{35}S]-sulfur trioxide trimethylamine complex + 19 mg of unlabelled complex.*
- ■ *200 mg PLE-L_d + about 1 mg of [^{35}S]-sulfur trioxide trimethylamine complex + 19 mg of unlabelled complex.*
- ▲ *blank experiment (without polyelectrolyte).*

procedure was about 14% for PLE-H_d and about 9% for PLE-L_d. The stability of the [^{35}S]-labelled polyelectrolytes was also examined by gel filtration. The elution pattern for a reaction mixture applied to the column immediately after resulfation is shown in Figure 6.



● *200 mg PLE-H_d + about 1 mg of [^{35}S]-sulfur trioxide trimethylamine complex + 19 mg of unlabelled complex.*

○ *blank experiment (without polyelectrolyte).*

C_a: fractions 6-10

C_b: fractions 14-24

FIGURE 2

Gel filtration of a mixture of [^{35}S]-labelled polyelectrolyte and [^{35}S]-containing low molecular weight compounds, using a Sephadex G·25 column

Two peaks were found, corresponding with [^{35}S]-labelled polyelectrolyte (C_a) and with low molecular weight products containing ^{35}S (C_b). The yield of the labelling procedure, expressed as:

$$\frac{\text{radioactivity in } C_a}{\text{radioactivity in } (C_a+C_b)} \times 100\% \quad \text{was 12.1\%.}$$

When samples of [^{35}S]-labelled polyelectrolyte (after dialysis or gel filtration) were applied to the column, only one peak was found corresponding with C_a, even after standing for more than 24 hours. When samples of the blank experiment (without polyelectrolyte) or samples of C_b were applied to the column, only one peak was found corresponding with C_b.

From these results it can be concluded that partial desulfation of

the polyelectrolytes, followed by resulfation with a [^{35}S]-sulfur trioxide trimethylamine complex, results in the formation of [^{35}S]-labelled polyelectrolytes, which show no release of the label in aqueous solutions.

The specific activity of the polyelectrolytes is about 0.2 μ Ci/mg, which is sufficiently high for the detection of a polyelectrolyte graft layer, containing [^{35}S]-labelled polyelectrolyte.

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