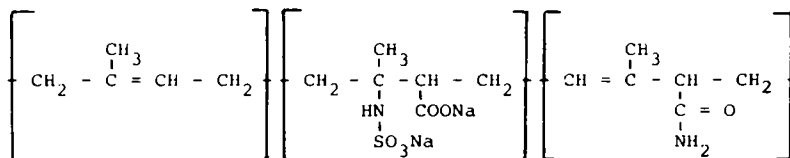


Comparison of Two Carboxylate-Activating Agents for the Modification of a Synthetic Heparinoid Polyelectrolyte

Introduction

Recently our group published an article (1) on the preparation and properties of a polyelectrolyte, showing anticoagulant activity. This polyelectrolyte (PLE) was derived from cis-1,4-polyisoprene and contains the following structural units:



In an attempt to improve the blood compatibility of polymeric materials, PLE has been linked to different surfaces either by means of cationic coupling agents (2) or covalently by making use of the residual double bonds in the polyelectrolyte (3). Immobilization of PLE onto polymeric surfaces can also be achieved by reaction of carboxylate groups of PLE with suitable functional groups, present on the polymeric surface. However, derivatization of the carboxylate groups might have a negative effect on the anticoagulant activity (4). Ebert et al. (5) observed a decrease of heparin anticoagulant activity, after derivatization of carboxylate groups present in heparin. This article describes the derivatization of the carboxylate groups, using two carboxylate-activating agents: Woodward's reagent K (WWRK) (N-ethyl-5-phenyl isoxazolium-3'-sulfonate) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), in order to study the effect on the anticoagulant activity and to find the optimal conditions for the immobilization of PLE.

Experimental

Materials

Polyelectrolytes: Polyelectrolytes PLE-H and PLE-L were synthesized using the method described previously (7, 8). The starting material was cis-1,4-polyisoprene (Cariflex IR 307, Shell), $\bar{M}_n = 270,000$, $\bar{M}_w/\bar{M}_n = 5.3$, for PLE-H and cis-1,4-polyisoprene, $\bar{M}_n = 6700$, $\bar{M}_w/\bar{M}_n = 1.1$, for PLE-L (7).

Anal. PLE-H: C, 35.87%; H, 5.34%; N, 5.29%; S, 9.69%. PLE-L: C, 36.39%; H, 5.38%; N, 5.47%; S, 9.91%.

Chemicals: Glycine, n-butylamine, and WWRK (N-ethyl-5-phenyl isoxazolium-3'-sulfonate) were purchased from Sigma. p-Nitro-benzylamine hydrochloride and EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] were purchased from Aldrich Europe.

Radiochemicals: [$2\text{-}^3\text{H}$] Glycine (15.0 Ci/mmol, 5 μg in 1 mL of 0.1M HCL) was purchased from New England Nuclear and [$1\text{-}^3\text{H}$] ethan-1-ol-2-amine hydrochloride (ethanolamine hydrochloride) (23 Ci/mmol, 4 μg in 1 mL of water) was purchased from the Radiochemical Centre Amersham (UK).

Dialysis tubing: Benzoylated dialysis tubing (Sigma D 7884) was used for the dialysis of solutions of PLE-L, and unmodified dialysis tubing (Cenco) was used for the dialysis of solutions of PLE-H.

Methods

Reaction of PLE with amines:

Activation of PLE with WWRK: To solutions of PLE in water or 0.1M phosphate-buffered saline (PBS) a solution of the amine (dissolved in the same solvent as PLE) was added. The mixture (pH 5-7.5) was cooled to 10°C, followed by the addition of WWRK (as a powder or as a freshly prepared solution in water) and the pH was readjusted to the desired value. For coupling of glycine, PLE was first activated with WWRK, the resulting solution was dialyzed for 3 \times 1 h against 0.1M PBS (to remove unreacted WWRK), and the glycine solution was added. The reaction was carried out at 10°C at the desired pH over a period of 16-24 h.

Activation of PLE with EDC: To solutions of PLE in water a solution of the amine was added. EDC was added as a powder or as a freshly prepared solution in water. The reaction was carried out at 20°C during 4-20 h (pH 2-7).

After the coupling reaction, the reaction mixtures were dialyzed against 0.1M PBS or 1M NaCl, and water. When radioactive compounds were used, the dialysate was sampled regularly to measure the radioactivity, and after each sampling the dialysate was replaced immediately by a fresh solution. The modified polyelectrolytes were isolated by freeze-drying of the solutions.

Radioactivity measurements: The radioactivity of [^3H] glycine-containing compounds was measured using a 2-mL sample and 10 mL of "Lumagel" scintillation medium. Each sample contained 0.5 mL of 0.1M PBS and 1.5 mL of water. The radioactivity of [^3H] ethanolamine-containing compounds was measured using 1 mL of liquid sample and 10 mL of scintillation medium. Each sample contained 0.5 mL of 1M NaCl and 0.5 mL of water. The samples were counted in a Packard Tricarb 2650 liquid scintillation counter.

Conductometric titrations: Conductometric titrations were carried out to determine the amount of carboxylate groups in the (modified) polyelectrolytes. PLE (100 mg) was dissolved in 150 mL of deionized water and an excess of NaOH was added to bring all carboxyl groups in the ionic form. The carboxylate groups were titrated by means of 0.064 M HCl.

Spectroscopy: Infrared (IR) spectra were recorded with a Beckman-TR-33 IR

TABLE I
Conductometric Titrations and Anticoagulant Activity
of Modified Polyelectrolytes

% of COOH maximal activated	mmol COONa/g		IU/mg \pm SD (n = 6) ^b	
	PLE-H ^a	PLE-L ^a	PLE-H	PLE-L
0	3.18 ^a	2.97 ^a	10.2 \pm 0.9	9.2 \pm 0.4
7	2.92	2.54	9.1 \pm 0.9	7.4 \pm 0.2
14	2.74	2.30	8.1 \pm 0.8	6.7 \pm 0.3
27	2.59	2.14	7.9 \pm 0.5	6.4 \pm 0.2
46	2.14	1.76	5.7 \pm 0.3	5.2 \pm 0.4
73	1.84	1.44	4.7 \pm 0.4	4.3 \pm 0.3
100	1.49	1.15	3.8 \pm 0.5	3.8 \pm 0.4

^a Calculated from % S (elemental analysis): 3.03 mmol COONa/g (PLE-H) and 3.10 mmol COONa/g (PLE-L), assuming mmol COONa/g = mmol NHSO₃Na/g.

^b As determined by APTT assay in conjuncture with heparin calibration curve.

spectrophotometer and ultraviolet (UV) spectra were recorded on a Zeiss-PM-6 UV spectrophotometer. Solutions of PLE in water with a concentration between 0.2 and 0.4 mg/mL were used for the UV analysis. Double-distilled water was always used as a blank.

Elemental analyses: Elemental analyses were performed by the Organic Chemical Institute TNO, Utrecht. The absolute error in the weight percentages of the elements was 0.2%.

Anticoagulant activity: Activated partial thromboplastin times (APTT) of bovine plasma were measured to study the effect of modification of the polyelectrolytes on the activation of the intrinsic coagulation (9). An increase of the APTT value is an indication of deficiencies of plasma coagulation factors or the presence of an anticoagulant. Tests were carried out according to the method described elsewhere (10). Results are expressed in international heparin units (IU/mg).

Results and Discussion

In order to explore the possibility of modification of carboxylate groups in PLE, coupling experiments were carried out with n-butylamine, using WWRK as carboxylate-activating agent (pH 6.5). In Table I it is shown that carboxylate

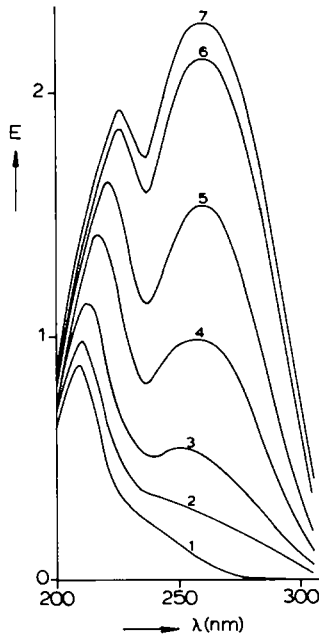
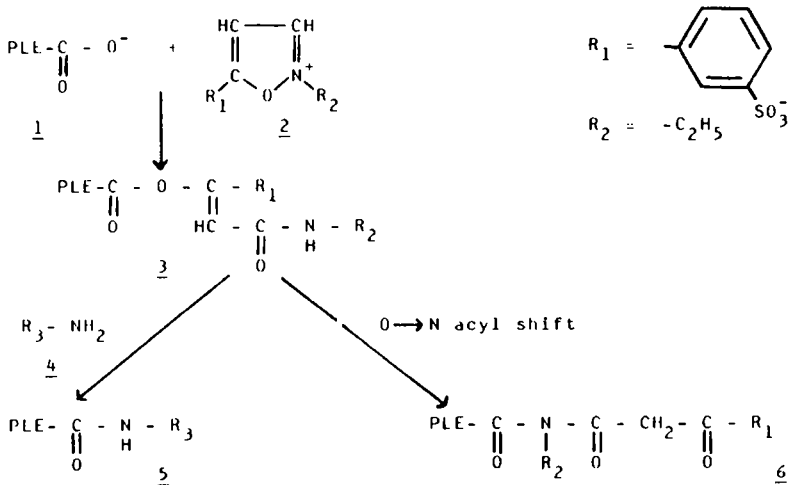


Fig. 1. UV spectra of butylamine-modified polyelectrolytes, derived from PLE-H. Percent of carboxylate groups maximal activated with WWRK: (1) 0, (2) 7, (3) 14, (4) 27, (5) 46, (6) 73, and (7) 100.

groups of PLE were modified, which obviously resulted in a decrease of the anti-coagulant activity. However, it cannot be concluded directly from these results that n-butylamide groups were formed (see scheme 1).



Scheme 1. WWRK coupling reactions with PLE and amine (11-13).

TABLE II

Coupling of [³H]-Glycine to PLE, Using WWRK as
Carboxylate-Activating Agent

pH	activation time (hrs) ^a	glycine mmol	% of original radioactivity in dialysis bag ^d	
			PLE-L	PLE-H
6.5	5	b	0.1 ^e	0.1 ^e
6.5	5	b	22.2	21.6
6.5	5	1.5 × 10 ⁻² ^b	0.8	0.4
7.5	5	c	0.2	0.2
6.5	1/6	c	9.4	7.4
6.5	2	c	10.4	9.6
6.5	7	c	13.4	18.1

^a 100 μ L of a WWRK solution (c = 0.15 mmol/mL) was added to 1 mL of PLE solution (c = 100 mg/mL 0.1M PBS) (maximal 5% of carboxylate groups of PLE activated).

^b 2×10^{-7} mmol [³H]-glycine added to 100 mg of PLE.

^c 7×10^{-7} mmol [³H]-glycine added to 100 mg of PLE.

^d After dialysis against 0.1M PBS during 110 h.

^e Blank experiment, without WWRK.

The mode of action of isoxazolium salts is well established (11-13). Carboxylate groups in PLE (1) can react with WWRK (2) to give intermediate 3. In the presence of an amine (4) a polyelectrolyte with amide groups might be formed (5), in competition with the rearrangement of 3 into 6. Because of the presence of aromatic groups in structures 3 and 6, UV spectra should give information about the modified structural units in the polyelectrolyte. It is obvious from the data, presented in Figure 1, that the absorption at 260 nm has to be attributed to the presence of structures 3 and/or 6 in the polyelectrolytes.

In order to investigate whether in the reaction of PLE with WWRK and amines not only activated esters and imides had been formed, but also coupling of amines with carboxylate groups had occurred, experiments were carried out with a radioactive amine.

[³H]-glycine was selected, and because glycine contains carboxylate groups, the activation and coupling reaction had to be carried out separately. The reaction conditions and the results of the coupling reactions are summarized in Table II. After dialysis of the reaction mixture for approximately 20 h, the radioactivity in the dialysis bag remained nearly constant, which means that the radioactive glycine had been stably incorporated in the polyelectrolytes. From

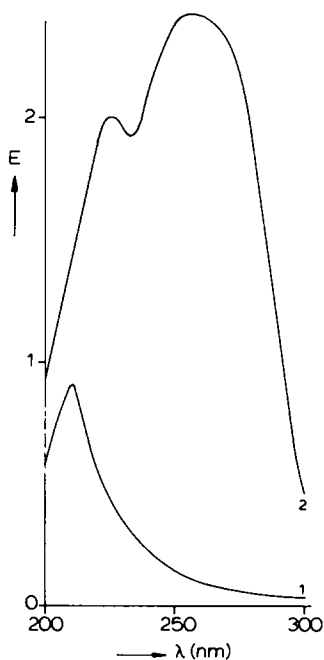


Fig. 2. UV spectra of modified polyelectrolytes derived from PLE-H. WWRK was added to give an activation of maximal 100% of the carboxylate groups. $C_{\text{PLE-H}} = 20 \text{ mg/mL}$ of 0.1M PBS, molar ratio amine/activated carboxylate groups (maximal) = 4:1. (1) PLE-H (no activation), PLE-H + WWRK (pH 7.5), PLE-H + WWRK + n-butylamine (pH 7.5); (2) PLE-H + WWRK (pH 6.5), PLE-H + WWRK + n-butylamine (pH 6.5).

the results it can be concluded that WWRK-activated carboxylate groups in the polyelectrolytes react with amines. There is a slight influence of the activation time, whereas the effect of the pH is more pronounced.

To investigate whether the low yield at pH 7.5 was due to the absence of activated carboxylate groups, additional coupling reactions were carried out with n-butylamine and PLE-H at pH 7.5. It can be concluded that no activation of carboxylate groups had occurred, because absorption at 260 nm was absent (Fig. 2). Contrary to this behavior, activation of carboxylate groups as well as coupling with amines did occur at pH 6.5.

The activated carboxylate and/or imide groups are rather stable, because they are still present in the modified polyelectrolytes after dialysis against 0.1M PBS and water for three days. Conversion of the excess of these groups into carboxylate groups would be beneficial, because carboxylate groups have been reported to be essential for the anticoagulant activity of the polyelectrolytes (4).

In order to study the regeneration of the carboxylate groups, a polyelectrolyte (PLE-H) was modified by addition of WWRK in such a quantity to give a maximal activation of 100% of the carboxylate groups (no amine added). This

TABLE III

Analytical Data of PLE-H and Derivatives

PLE	C/N ^a	N/S ^a	APTT	SD	n	mmol ^a COONa/g ^c	
			(sec) ^b				
PLE-H	7.91	1.25	157.4	2.1	3	3.03	3.18
PLE-H-W-b	7.56	1.31	149.4	2.1	3	2.72	2.86
PLE-H-W	-	-	76.5	2.2	6	-	1.23

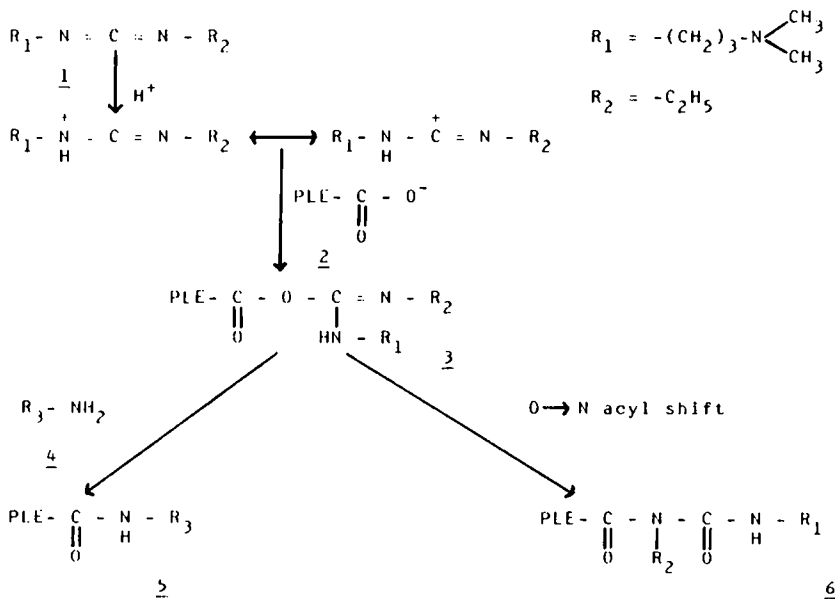
^a Calculated from elemental analysis data.

^b APTT of blank (without PLE): 57.1 s, SD = 7.7, n = 65.

^c Determined by conductometric titration.

modified polyelectrolyte (PLE-H-W) was brought into 0.05M NaOH for 24 h at 20°C, followed by dialysis against water for three days. The UV spectrum of the base-treated polyelectrolyte (PLE-H-W-b) was identical to the UV spectrum of PLE-H. Also, the IR spectrum of PLE-H-W-b was identical to that of PLE-H, whereas in the IR spectrum of PLE-H-W additional absorption lines were present at 1740 and 1120 cm⁻¹.

In Table III analytical data of PLE-H and derivatives are given. From the



Scheme 2. Carbodiimide coupling reactions with PLE and amine (14).

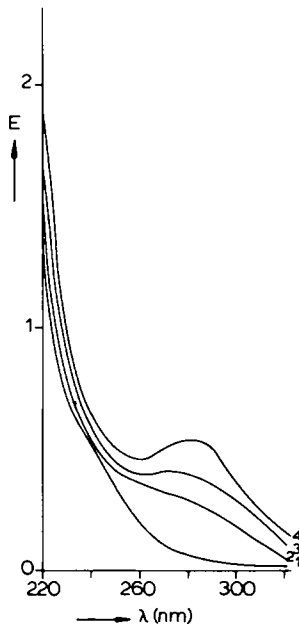


Fig. 3. UV spectra of modified polyelectrolytes derived from PLE-H. Carboxylate groups were activated with WWRK or EDC to a maximum of 50%. $C_{\text{PLE}} = 15 \text{ mg/mL}$ of water, molar ratio amine/activated carboxylate groups (maximal) = 4:1. Coupled amine: p-nitrobenzylamine. All products treated with base. (1) PLE-H (no activation), PLE + WWRK or EDC (pH 6.5), PLE-H + WWRK + amine (pH 5 and 6.5), PLE-H + amine (pH 6.5); (2) PLE-H + EDC + amine (pH 2); (3) PLE-H + EDC + amine (pH 5); (4) PLE-H + EDC + amine (pH 6.5).

results in Table III it can be concluded that after derivatization of the polyelectrolyte, using WWRK as the carboxylate-activating agent, the carboxylate groups, which were modified but not used for coupling with amine, can be regenerated by a base treatment, thereby minimizing the loss in anticoagulant activity of the polyelectrolyte.

Coupling reactions were also carried out with EDC as the carboxylate-activating agent (and WWRK for comparison). In scheme 2 the pathway for carbodiimide-facilitated coupling reactions is shown. p-Nitrobenzylamine was used as amine and because of the presence of the p-nitrobenzyl group, the modified polyelectrolytes will show an absorbance at 270 nm, when coupling occurs.

From Figure 3 it can be seen that the reaction with p-nitrobenzylamine and EDC resulted in modified polyelectrolytes with an absorbance at 270 nm, indicating a coupling with p-nitrobenzylamine. Using WWRK as the activating agent, modified polyelectrolytes were obtained, which did not show an absorbance at 270 nm as a result of the low coupling yield. Also, from IR spectroscopy the presence of p-nitrobenzylamine groups in the EDC-modified polyelectrolytes could be shown (peaks at 1520 and 1340 cm^{-1}).

TABLE IV

Coupling of [^3H]-Ethanolamine to PLE-L, Using EDC as
Carboxylate-Activating Agent

Maximal % of COOH acti- vated (COOH $^{\ominus}$)	molar ratio COOH $^{\ominus}$ /amine $^{\text{b}}$	C _{PLE} in reaction mixture $\mu\text{g/ml}$	pH	% of original radioactivity in dialysis bag $^{\text{c}}$
-	-	24	6	< 0.1
5	1.0	24	5	0.5
5	1.0	24	6	1.0
5	1.0	24	7	1.2
-	-	159	6.5	< 0.1
5	~ 4000	159	6.5	13.7
5	2.0	159	6.5	2.6
5	1.0	159	6.5	1.8
10 $^{\text{a}}$	~ 40	435	6.5	92.4

$^{\text{a}}$ EDC added as a powder.

$^{\text{b}}$ 10 μL of [^3H]-ethanolamine solution was added to the PLE solution.

$^{\text{c}}$ After dialysis against 1M NaCl and water for 100 h.

A complication that arises with the use of EDC as an activating agent is the occurrence of side reactions (scheme 2), as was also observed with WWRK (scheme 1). All EDC-modified polyelectrolytes showed an absorbance at 1740 cm^{-1} , due to the presence of 6. Base treatment, however, did not result in regeneration of carboxylate groups, in contrast to what was found with the WWRK-modified polyelectrolytes. Coupling, using EDC as the activating agent, was also studied with a labeled amine ([^3H]-ethanolamine). Reaction conditions and results are shown in Table IV. The data in Table IV show that coupling with ethanolamine occurs, using EDC as the carboxylate-activating agent. By choosing the proper reaction conditions, rather high coupling yields can be obtained.

In summary it can be concluded that WWRK as well as EDC can be used for modification of carboxylate groups of the polyelectrolytes. However, the coupling yield, using WWRK, is low. The carboxylate groups, which were modified but not used for the coupling with amine, can be regenerated by a simple base treatment. With EDC much higher coupling yields can be obtained, but a complication is the occurrence of a side reaction, leading to the formation of stable imide groups in the polyelectrolytes.

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L. C. Sederel
L. van der Does
T. Beugeling
J. Feijen
A. Bantjes

Department of Chemical Technology
Biomaterials Section
Twente University of Technology
P.O. Box 217
Enschede, The Netherlands

S. W. Kim

Department of Pharmaceutics
College of Pharmacy
Skaggs Hall
University of Utah
Salt Lake City, Utah 84112

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