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4-Nitrophenyl chloroformate activation of poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide] and poly-α,β-[*N*-(2,3-dihydroxypropyl)-D,L-aspartamide]

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Poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide] (PHEA) and poly-α,β-[*N*-(2,3-dihydroxypropyl)-D,L-aspartamide] (PDHPA) are suitable as macromolecular drug carriers. In order to introduce amine-containing drug moieties onto these polymers, partial conversion of the hydroxyl side groups is required. This paper describes the 4-nitrophenyl chloroformate activation of PHEA and PDHPA. It is shown that, during the course of the activation of PHEA, only linear aromatic carbonate structures were formed. However, during the activation reaction of PDHPA, conversion of 4-nitrophenyl carbonates into cyclic carbonate structures could be observed. The relative amount of the two types of carbonate moieties could be controlled by addition of the appropriate catalyst. During the activation reaction of both polymers, the total content of carbonate groups could, for a given set of reaction conditions, be controlled by the amount of chloroformate added. The 4-nitrophenyl carbonate groups easily reacted with amines. However, the conversion of the cyclic carbonate moieties into urethane-bound derivatives was only possible with highly reactive amines. This could be an interesting property for the introduction of different types of amine-containing derivatives onto the polymer backbone. The results of this study demonstrate the feasibility of the described activation methods to prepare macromolecular prodrugs.

(Keywords: poly(aspartamides); chloroformate activation; carbonates)

INTRODUCTION

The coupling of biologically active compounds to soluble polymeric carriers is an attractive approach to advanced drug delivery. The polymeric carrier may enhance the stability of the attached ligand, lower its toxicity and antigenicity, slow down its elimination, or promote selective deposition in the body[1–3].

In such polymeric derivatives the drug is covalently linked onto the polymer backbone directly or via a spacer group. Following administration, the active agent can be released as a result of hydrolytic or enzymatic cleavage of chemical bonds.

Derivatives of poly(α-amino acids) have been suggested as potential biomedical polymers, plasma expanders, or drug delivery systems[4–6]. The first poly(α-amino acid) proposed as a potential plasma expander was the sodium salt of poly(glutamic acid). This macromolecule, however, proved to be toxic[7]. Subsequently, interest was focused on the hydrophilic uncharged derivative poly-[*N*-(2-hydroxyethyl)-L-glutamine] (PHEG), which was proved to be efficient, non-toxic and non-immunogenic in test animals[8]. However, large-scale production of PHEG raised complex technical and economic problems, which seemed to justify research on other polymers that were easy to produce at low cost. In this respect, the synthesis of analogous derivatives of poly(aspartic acid) appeared to be of particular interest[5,9–12]. The water-soluble poly-α,β-[N-(2-hydroxyethyl)-D,L-aspartamide] was originally proposed as a plasma expander by Neri[5] and then extensively studied as a drug carrier in pharmaceutical controlled-release systems[12–15]. The hydroxyl moieties present in this polymer permit binding to carboxyl- or amine-containing drug compounds, producing a water-soluble macromolecular adduct. Complete solubility in water is a much-desired feature of polymer–drug conjugates for intravenous administration. In this work, poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide] (PHEA) and poly-α,β-[*N*-(2,3-dihydroxypropyl)-D,L-aspartamide] (PDHPA) (*Figure 1*), containing respectively one or two hydroxyl functions per monomer unit, were selected as drug carriers.

For the coupling of drug molecules to these hydroxyl-containing polymers, a previous transformation of the latter into suitable reactive derivatives is generally required. An attractive activation procedure reported in the literature is the chloroformate activation method[16–20]. In this work, the 4-nitrophenyl chloroformate activation of PHEA and PDHPA was investigated. The influence of the applied reaction conditions (solvent, catalyst) on the obtained degree of activation was determined. Moreover, the nature of the active

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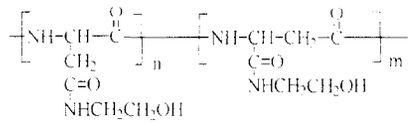
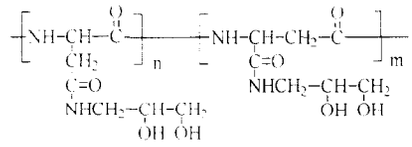


PHEA



PDHPA

Figure 1 Structural formulae of PHEA and PDHPA

carbonate groups introduced by chloroformate activation was studied. Finally, the reaction of the activated polymer with amines was checked. In order to demonstrate the usefulness of the carbonate-substituted derivatives for coupling of amine-containing drug moieties, 1-amino-2-propanol and glycine methyl ester were used as model compounds.

EXPERIMENTAL

All chemicals were purchased from Janssen Chimica (Beerse, Belgium). Solvents were dried and purified by fractional distillation. ^{1}H n.m.r. spectra were recorded on a Brucker WH 360 spectrometer.

Preparation of the polymers

Synthesis of poly(succinimide). D,L-Aspartic acid (25 g, 187 mmol) was mixed with 10 g of 85% phosphoric acid (87 mmol). Polymerization was carried out at constant temperature (150–180°C) under reduced pressure for 2.5 h. The reaction mixture was dissolved in 100 ml *N*,*N*-dimethylformamide (DMF) and precipitated in 500 ml of water. The product was filtered and washed with an excess of water and finally dried at 110°C over phosphorus pentoxide.

Synthesis of PHEA and PDHPA. As an example, the synthesis of PHEA is described. First, 10 g (103 mmol) of poly(succinimide) was dissolved in 60 ml of DMF. The reaction mixture was stirred and 15 ml of 2-aminoethanol was added dropwise. The reaction temperature was kept at 25–30°C for 2 h. After neutralization of the excess amine with acetic acid, the reaction mixture was precipitated in 300 ml of dry ethanol. PHEA was obtained as a white solid after filtration and purification by preparative g.p.c. (Sephadex G25, water as eluent). PDHPA was prepared under the same reaction conditions using 2,3-dihydroxypropylamine instead of 2-aminoethanol.

The polymers were characterized by n.m.r. analysis (D_2O). PHEA: δ = 2.7–2.9 ppm (β-CH_2), 3.3 ppm (C(O)NH–CH_2), 3.7 ppm (CH_2–OH), 4.7 ppm (α-CH); PDHPA: δ = 2.7–2.9 ppm (β-CH_2), 3.1–3.4 ppm (C(O)NH–CH_2), 3.45–3.65 ppm (CH_2–OH), 3.8 ppm (CH–OH), 4.7 ppm (α-CH).

The molecular weight of the polymers was determined by analytical g.p.c. (Tessek HemaBio 1000, 300, 100 and 40; citrate buffer pH = 6, 0.1 M; r.i. detection; calibration by dextran standards 123 600, 66 700, 43 500, 21 400, 9990, 4440). PHEA: M_w = 53 000, M_n = 23 500; PHDPA: M_w = 46 300, M_n = 20 400.

4-Nitrophenyl chloroformate activation of PHEA

The activation of PHEA in optimal reaction conditions is given as an example. First, 2 g of PHEA (12.5 meq, freeze dried in the reaction flask) was dissolved in a mixture of 33 ml of 1-methyl-2-pyrrolidinone (NMP) and 9 ml of pyridine. Then, 0.346 g (2.8 mmol) of 4-dimethylaminopyridine (DMAP) and 3.78 g (18.9 mmol) of chloroformate were added at 0°C. At regular time intervals, 5 ml aliquots were withdrawn from this reaction mixture and added to 40 ml of an anhydrous diethyl ether/acetone mixture (60/40, v/v). The white precipitate was collected by filtration and washed five times with the same mixture. The product was finally dried under reduced pressure.

Determination of the 4-nitrophenyl carbonate content

Activated polymer (5 mg) was added to 10 ml of 0.1 M sodium hydroxide. The concentration of the sodium 4-nitrophenolate was determined u.v. spectrometrically (λ_M = 402 nm, ε_M = 18 400 l mol^{-1} cm^{-1}).

Determination of the total carbonate content

First, 75 mg of activated polymer was added to 10 ml of 0.1 M barium hydroxide. The mixture was then refluxed for 1 h, while care was taken to prevent uptake of CO_2 from the air. After cooling to room temperature, the reflux condenser was washed with 10 ml of CO_2-free water and the aqueous solution was titrated with 0.1 M HCl using phenolphthalein as indicator.

4-Nitrophenyl chloroformate activation of PDHPA

The activation of PDHPA using DMAP as a catalyst is described as an example. First, 2 g of PDHPA (10.6 meq, freeze dried in the reaction flask) and 0.192 g (1.6 mmol) of DMAP were dissolved in a mixture of 56 ml NMP and14 ml pyridine. Then, 3.17 g (15.8 mmol) of chloroformate was added stepwise to the reaction mixture at 0°C. At regular time intervals, 5 ml aliquots were withdrawn from this reaction mixture and added to 40 ml of an anhydrous diethyl ether/acetone mixture. The product was finally dried under reduced pressure.

Reaction of the activated polymers with amines

The reaction of activated PHEA with 1-amino-2-propanol is given as an example. First, 0.5 g of activated polymer (3.16 mmol, 0.411 mmol carbonates: 13% substituted) was dissolved in a mixture of 10 ml DMF and 1 ml pyridine. Then, 37.5 mg (0.5 mmol) of 1-amino-2-propanol was added. Stirring was continued for 12 h. The reaction product was isolated by precipitation in excess diethyl ether/acetone (70/30, v/v) and purified by preparative g.p.c. using water as eluent (Sephadex G25). After freeze drying, the product was obtained as a white solid.

Determination of the degree of amine substitution

The 360 MHz ^{1}H n.m.r. spectrum was taken in D_2O. The degree of amine substitution was calculated from the comparison of the signals characteristic of the polymer backbone (δ = 2.7–2.9 ppm, β-CH_2) and the signal

characteristic of the amine: for 1-amino-2-propanol, $\delta = 1.1$ ppm, CH_3; for glycine methyl ester, = 1.25 ppm, OCH_3.

RESULTS AND DISCUSSION

Activation of PHEA with 4-nitrophenyl chloroformate

It has been demonstrated before that hydroxyl-containing polymers can be converted into reactive derivatives by reaction with 4-nitrophenyl chloroformate[18–20]. In this study the same method was applied for the activation of PHEA and PDHPA.

4-Nitrophenyl chloroformate-activated PHEA (*Figure 2*) was initially prepared in *N*,*N*′-dimethylformamide (DMF)/pyridine at 0°C[21].

The content of 4-nitrophenyl carbonate moieties was determined during the course of the reaction. The results obtained indicate that the reaction proceeds slowly. The efficiency of the reaction is very low (Table 1). Even addition of the better catalyst 4-dimethylaminopyridine (DMAP) only slightly increased the amount of 4-nitrophenyl carbonate substitution. This is in agreement with literature data for the activation of poly[*N*-(2-hydroxypropyl)methacrylamide] with 4-nitrophenyl chloroformate in DMF/pyridine[21].

Plausible explanations for this low yield may be hydrolysis of the chloroformate catalysed by pyridine or DMAP, or other side reactions such as interaction between the chloroformate and DMF. Hall[22] and Horning[23] reported the formation of complexes during reaction of DMF with chloroformates (*Figure 3*). Moreover, Pattison[24] studied the reaction of aryl chloroformates and DMF and demonstrated the formation of aryloxy-substituted immonium salts (*Figure 3*).

Therefore, the reaction between DMF and 4-nitrophenyl chloroformate was investigated. ^1H n.m.r. analysis demonstrated the formation of an adduct. The formation of an immonium salt after reaction of the chloroformate and DMF was proved by n.m.r. analysis ($CDCl_3$): $\delta = 2.9$, 3.1 ppm ($2 \times CH_3$ of DMF), 2.5, 2.8 ppm ($2 \times CH_3$ of the immonium salt), 7.0, 8.0 ppm (aromatic protons of 4-nitrophenol), 7.9, 8.2 ppm (aromatic protons of the immonium salt), 10.8 ppm (CH of the immonium salt). After 2 h of reaction at 0°C, 10% of the chloroformate was converted into the immonium salt.

Table 1 Degree of 4-nitrophenyl carbonate substitution on PHEA as a function of the amount of chloroformate added in DMF as a solvent

Added chloroformate (mole per monomer unit)	Degree of activation (mol%)	
	Pyridine	DMAP
0.5	2.5	2.5
1	8.0	10.0
1.5	12.5	17.0

Based on these results, it was decided to select an alternative solvent. Several authors proved that the reaction between amide and chloroformate is limited for *N*-methylpyrrolidone (NMP) as a solvent[22,25]. Again, the stability of the chloroformate in this solvent was determined by n.m.r. spectroscopy: even after 24 h of reaction no reaction product could be observed. Therefore, NMP was used as a solvent for the 4-nitrophenyl chloroformate activation of PHEA and PDHPA.

4-Nitrophenyl carbonate-substituted PHEA derivatives were prepared by treatment of the polymer with the chloroformate in NMP/pyridine at 0°C, in the presence or absence of 3 mol% of DMAP as a catalyst. The content of aromatic carbonate groups (mol%) was determined during the course of the reaction (*Figure 4*).

These results clearly indicate the significant influence of the addition of DMAP on the degree of activation. The content of reactive carbonate moieties initially

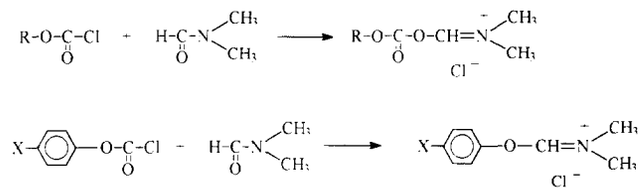


Figure 3 Reaction of DMF with chloroformates

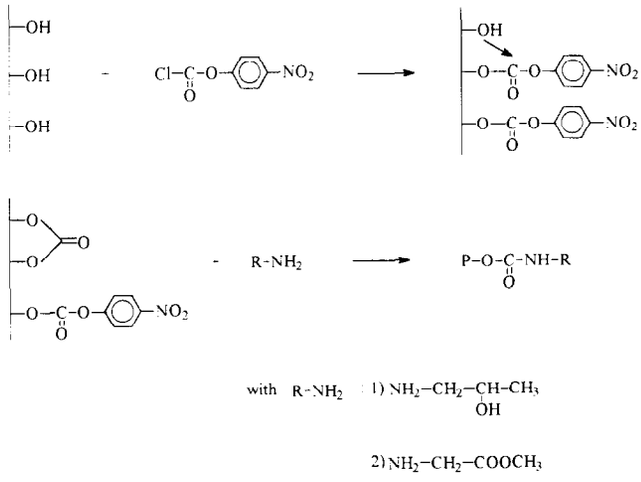


Figure 2 Reaction of PHEA (P–OH) with 4-nitrophenyl chloroformate, conversion of linear aromatic carbonate structures into cyclic carbonate moieties and further reaction of 4-nitrophenyl carbonate-substituted PHEA with model amines

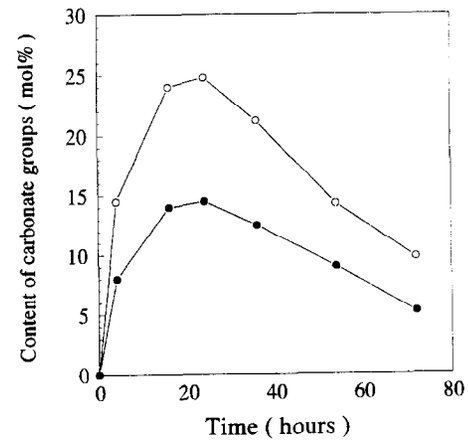


Figure 4 Degree of 4-nitrophenyl carbonate substitution on PHEA during the course of the reaction, in the presence or absence of DMAP as a catalyst: (●) reaction of PHEA with chloroformate in absence of DMAP; (○) reaction of PHEA and chloroformate in presence of 3 mol% DMAP

increases, reaches a maximum value and then slowly decreases. Plausible explanations for this decrease are slow hydrolysis of the reactive species, or reaction of the aromatic carbonates with polymeric hydroxyl groups with the formation of cyclic carbonate structures as reported for the activation of dextran and pullulan[18,19]. In order to examine whether hydrolysis or side reactions occurred, the total carbonate content was determined by hydrolysis with barium hydroxide according to the method of Kol'tsova[26]. The resulting data demonstrated that, at any stage of the reaction, the total carbonate content was equal to the content of 4-nitrophenyl units (*Table 2*).

From these results it can be concluded that no carbonate structures other than linear ones were formed. The decrease of reactive carbonate substitution after 24 h of reaction is probably due to slow hydrolysis. Since both solvents and catalyst were carefully dried, the water content of the polymer was checked. Thermogravimetric analysis proved that freshly freeze-dried PHEA contained only 2 wt% of water whereas the polymer that was stored in a desiccator for 6 months contained 8 wt% of water. Therefore, the activation experiments were repeated using PHEA that was freeze-dried in the reaction flask. Applying these new reaction conditions, the maximum degree of activation was observed after 1 h of reaction, after which the degree of activation remained constant for approximately 24 h (*Figure 5*).

Furthermore, the effect of the amount of chloroformate added on the degree of reactive carbonate substitution was investigated. PHEA was reacted with different amounts of chloroformate in NMP/pyridine (4/1, with 15 mol% of DMAP) at 0°C for 30 min. The results of this study are shown in *Figure 6*. These data demonstrate that the activation degree is proportional to the amount of chloroformate added. One can conclude that, using the appropriate reaction conditions, the activation reaction is fast, reproducible and the degree of introduced reactive species can be controlled by the amount of chloroformate added.

Activation of PDHPA with 4-nitrophenyl chloroformate

The 4-nitrophenyl chloroformate activation of PDHPA was studied at 0°C in NMP/pyridine (4/1, v/v) as a solvent. According to the literature, activation of a polyalcohol with chloroformates in the presence of strong bases often results in rearrangements of the linear aromatic carbonates with formation of cyclic carbonate derivatives[18,19] (*Figure 2*).

During the activation of PDHPA, having two hydroxyl functions per monomer unit, the rearrangement of linear carbonates to cyclic carbonate derivatives is probable. According to the literature[26-28], the formation of cyclic carbonate moieties is catalysed by strong bases. Therefore, the influence of the catalyst on the degree of aromatic and total carbonate substitution was studied. The aromatic carbonate content was determined by u.v. analysis and the total carbonate content was calculated after hydrolysis with excess barium hydroxide and titration with HCl.

Activation of PDHPA in the presence of pyridine as a catalyst. The 4-nitrophenyl chloroformate activation of PDHPA was first investigated in the presence of pyridine as acid acceptor. The results are given in *Figure 7*. These data indicate that cyclic carbonate structures were formed. The amount of cyclic carbonate groups is low at low activation degrees, but increases during the course of the reaction. The aromatic carbonate substitution is high for short reaction times but decreases for longer reaction times. The total carbonate content (expressed as the number of substituted units per 100 monomer units) rises continuously during the reaction and reaches a value of more than 100% after 24 h of reaction. This indicates that more than one hydroxyl function per monomer unit was substituted with an aromatic reactive group. These data show that during the reaction of PDHPA with chloroformate both linear and cyclic carbonate structures were formed. By adjusting the reaction time, the relative amount of carbonate groups can be varied from 88% linear carbonates after 30 min to 20% after 30 h of reaction.

Activation of PDHPA in the presence of DMAP as a catalyst. In the next set of experiments DMAP was added as a catalyst during the activation of PDHPA in NMP/pyridine. The course of the reaction is given in *Figure 8*. It is clear that both aromatic and cyclic carbonate contents are approximately constant as a function of time; the aromatic active species are slowly converted into cyclic carbonate moieties. Moreover, at any stage of the reaction, the amount of cyclic carbonate groups is significantly higher when DMAP is added as a catalyst. Furthermore, the influence of the amount of chloroformate added on the obtained activation degree was determined. *Figure 9* shows that the total carbonate content changes proportionally with the amount of chloroformate added. Similar data were obtained after 1 h and 4 h of reaction.

Activation of PDHPA in the presence of triethylamine as a catalyst. In the last experiment the activation was carried out in the presence of triethylamine (TEA) as a catalyst. *Figure 10* indicates that the results are similar to those using DMAP as a catalyst. However, the

Table 2 Determination of the aromatic and total carbonate contents of 4-nitrophenyl chloroformate-activated PHEA (NMP/pyridine, 15 mol% DMAP, 1.5 mol chloroformate per monomer unit)

Reaction time (h)	4-Nitrophenyl carb. content (mol%) (u.v. analysis)	Total carb. content (mol%) (barium hydroxide/HCl)
4	14.5	15.5
16	24.0	22.5
28	12.0	12.0
54	10.0	9.5
72	9.5	10.0

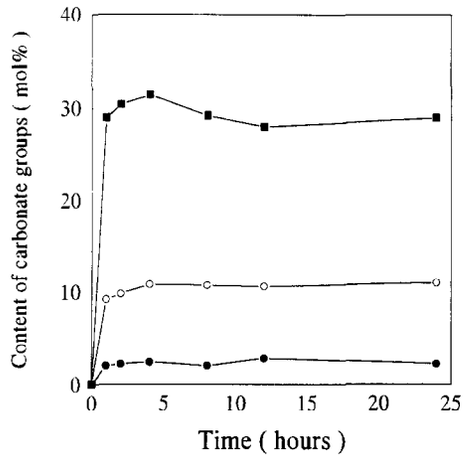


Figure 5 Degree of 4-nitrophenyl carbonate substitution on PHEA during the course of the reaction, for different amounts of chloroformate added (15 mol% of DMAP): (●) reaction of PHEA with 0.25 eq chloroformate per monomer unit; (○) reaction of PHEA with 0.75 eq chloroformate per monomer unit; (■) reaction of PHEA with 1.5 eq chloroformate per monomer unit

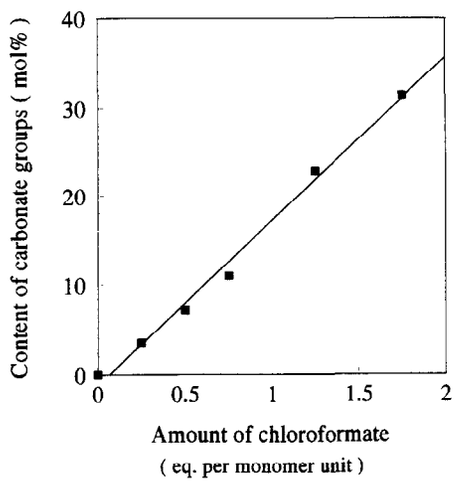


Figure 6 Degree of 4-nitrophenyl carbonate substitution on PHEA as a function of the amount of chloroformate added (15 mol% of DMAP)

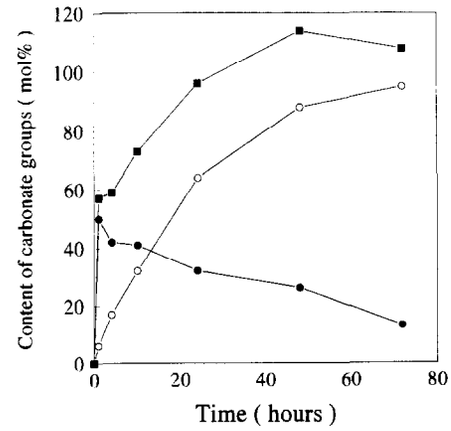


Figure 7 Degree of activation of PDHPA during the course of the reaction: (●) aromatic carbonate content; (○) cyclic carbonate content; (■) total carbonate content

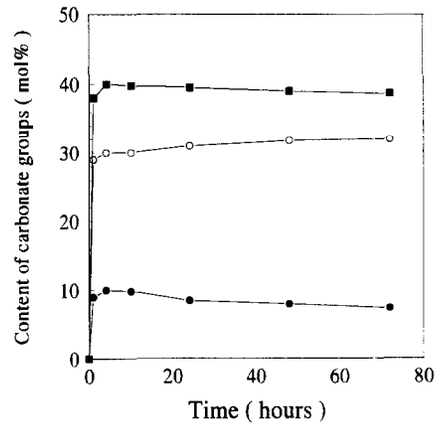


Figure 8 Degree of activation of PDHPA during the course of the reaction, using DMAP as a catalyst (15 mol%): (●) aromatic carbonate content; (○) cyclic carbonate content; (■) total carbonate content

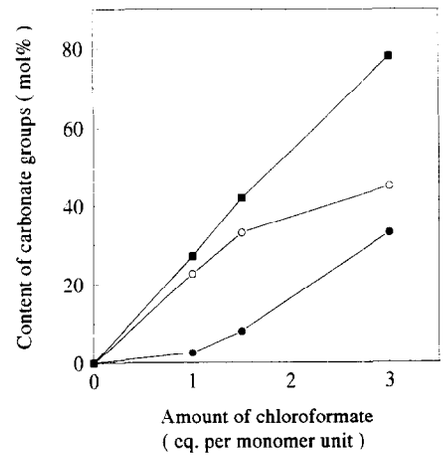


Figure 9 Degree of activation of PDHPA as a function of the amount of chloroformate added (15 mol% of DMAP): (●) aromatic carbonate content; (○) cyclic carbonate content; (■) total carbonate content

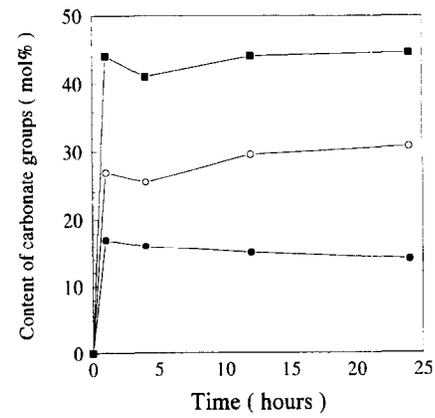


Figure 10 Degree of activation of PDHPA during the course of the reaction, using triethylamine as a catalyst (15 mol%): (●) aromatic carbonate content; (○) cyclic carbonate content; (■) total carbonate content

amount of linear carbonate groups is higher in the presence of TEA than in the presence of DMAP. Comparing the different reaction conditions, the highest substitution of linear aromatic carbonates was obtained using pyridine as a catalyst. The application of a catalyst with a higher basicity, such as DMAP and TEA, results in the formation of a higher degree of cyclic carbonate structures.

Finally the synthesis of polymeric derivatives carrying only cyclic carbonate moieties was investigated. In contrast to their 4-nitrophenyl carbonate-substituted analogues, these derivatives would be water-soluble even at high degrees of activation. Therefore, such activated polymers could be interesting for further applications. PDHPA derivatives containing only cyclic carbonate structures were successfully prepared by reaction of the starting polymer with 4-nitrophenyl chloroformate at 0°C in NMP/pyridine as solvent. After 1 h of reaction excess TEA or DMAP was added to the reaction mixture. The expected product was isolated after 30 min of reaction and proved to be a water-soluble conjugate.

These results indicate that, by choosing the appropriate catalyst, reaction time and amount of chloroformate added, activated PDHPA derivatives could be prepared with controlled degree of linear and cyclic carbonate substitution.

Reaction of 4-nitrophenyl carbonate-substituted PHEA with amines

Before studying the reaction of activated PHEA with amine-containing drug derivatives, the reaction with model amines 1-amino-2-propanol and glycine methyl ester was tested (*Figure 2*).

The reactive carbonate-containing PHEA was reacted overnight with the model amines in DMF/pyridine (9/1). The degree of substitution was calculated from n.m.r. analysis of the reaction product. The results are summarized in *Table 3*. These data demonstrate that, within experimental error, the degree of substitution is equal to the degree of activation of the polymer. This gives further evidence that during chloroformate activation only aromatic carbonate structures were formed.

Reaction of activated PDHPA with amines

Reaction of PDHPA with 4-nitrophenyl chloroformate leads to the formation of linear and cyclic carbonate structures. The reactivity of these carbonate moieties in reaction with amines was investigated, using 1-amino-2-propanol as model amine. The activated polymer was reacted with the amine overnight in DMF/pyridine. Complete conversion of both the aromatic (18 mol%) and the cyclic carbonate structures (35 mol%) into urethane-bound 1-amino-2-propanol (52 mol%) was demonstrated by n.m.r. analysis of the reaction product. Furthermore, i.r. analysis showed the absence of 4-nitrophenyl carbonate absorption (1795, 1580 cm^{-1}) and cyclic carbonate absorption (1785 cm^{-1}) and the presence of a urethane absorption (1695 cm^{-1}).

On the contrary, when glycine methyl ester was used as a model amine, only the linear aromatic carbonate structures were converted after 24 h of reaction. This phenomenon was proved by both n.m.r. and i.r. analysis. These data indicate that the reactivity of the amine group of the amino acid derivative is not sufficient to react with the cyclic carbonate structures. However, this could be an interesting property. Using this method, two types of amine derivatives could be introduced selectively onto the polymeric carrier: a less reactive amine reacting with the aromatic carbonate moieties and subsequently a second more reactive species opening the cyclic carbonate structures. To prove this hypothesis, activated PDHPA was reacted consecutively with glycine methyl ester and 1-amino-2-propanol respectively. N.m.r. analysis of the resulting product proved substitution of 18 mol% of glycine methyl ester (aromatic carbonate content) and 35% of 1-amino-2-propanol (cyclic carbonate content). This result shows the feasibility that two different types of amine-containing moieties (e.g. different types of drug derivatives) could be introduced along the polymeric carrier. Since the relative amount of linear and cyclic carbonate groups can be controlled, this activation procedure could be useful for the preparation of well-defined macromolecular drug conjugates.

Table 3 Extent of aromatic carbonate substitution in the activated PHEA calculated from u.v. analysis compared to the extent of amine substitution on the polymer calculated from n.m.r. analysis

Degree of activation (mol%)	1-Amino-2-propanol (mol%)	Glycine methyl ester (mol%)
6	7	6
13	12	14
19	20	18
28	30	31
33	33	34

CONCLUSION

The results of this study clearly demonstrate the feasibility of the 4-nitrophenyl chloroformate activation in the preparation of PHEA and PDHPA prodrugs. The degree of activation can be controlled by the amount of chloroformate added. The relative amount of aromatic and cyclic carbonate structures during the activation of PDHPA can be varied by applying the appropriate catalyst. Further, the coupling of activated PHEA with amines is quantitative. Also, the conversion of the different carbonate moieties in activated PDHPA can be complete by an appropriate selection of the amine compound or compounds. This indicates that the described method is useful for further coupling of amine-containing drug derivatives.

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