Studies of the esterification of dextran: Routes to bioactive polymers and graft copolymers

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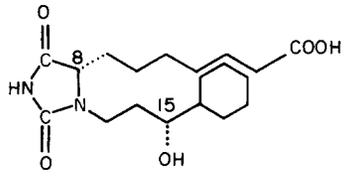
Problems associated with the esterification of dextran as a means of coupling bioactive molecules or introduction of functionality suitable for graft polymerization are considered. In particular, the importance of eliminating side-reactions which incorporate into dextran unwanted residues, e.g. groups containing nitrogen, is emphasized and practical techniques for minimizing this are described. We have developed a formamide-based solvent suitable for esterification with the aid of dicyclohexyl carbodiimide (DCC) and carbonyl di-imidazole (CDI) as coupling agents. The preferred catalyst is *p*-pyrrolidinopyridine. CDI has the advantage of enabling dimethylsulphoxide to be used as solvent for dextran and other hydroxylic polymers without inducing oxidation of hydroxyl groups. This coupling agent is flexible and offers a choice of two routes to esterification, each having its merits. We have optimized conditions for coupling by use of butyric acid as model. Esterification of dextran has been employed in the preparation of soluble bioactive macromolecules by coupling the anti-platelet agent (I) and also in the synthesis of graft copolymers via introduction of 2-bromopropionate groups. Crosslinking of dextran and the polymerization of dipyridamole have been effected by use of CDI.

(Keywords: dextran; esterification; bioactive macromolecules; graft copolymers; dicyclohexylcarbodiimide; carbonyl di-imidazole)

INTRODUCTION

As part of a study of the properties of anti-platelet agents attached to macromolecules[1] we have explored chemical routes[2-4] suitable for coupling (I) [5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxy-propyl)-hydantoin] to a series of polymers.



(I) (BW 245C)

Four procedures have been used: (i) synthesis of methacrylate esters of (I) for subsequent copolymerization; (ii) synthesis of halogen-containing esters of (I), which, in the presence of an appropriate metal carbonyl, are active initiators of polymerization and are suitable for preparing vinyl polymers having (I) as a terminal unit; (iii) direct chain-transfer to (I) in a free-radical polymerization and (iv) direct coupling by esterification of the carboxyl groups of (I) with hydroxyls in preformed polymers.

This paper is concerned with the last process, with particular reference to dextran.

For many years crosslinked dextran (sephadex) has been used for immobilization of active species in affinity chromatography. As would be expected, a large variety of coupling reactions have been used with sephadex, but few of the techniques have been employed to synthesize esters of dextran. For our purpose it was necessary to form an ester link to dextran, so that aqueous solvents were unsuitable. (Note that Simionescu *et al.*[5,6] claim to have synthesized ester links in media containing water with the aid of dicyclohexylcarbodiimide; however, they report that the process becomes less efficient as the water content is increased.)

The advantages of dextran as a macromolecular carrier for drug immobilization are well-accepted, as is apparent from a brief list of species which have been coupled to it. These include[7] insulin, organomercurials, detergents, n-alkyl chloroformates, daunomycin, amphetamine, proteins including haemoglobin, ampicillin, various dyes, novocaine. A variety of coupling agents and conditions has been used[7]. Reactions are frequently performed in aqueous solution often with the aid of cyanogen bromide[8], or periodic acid followed by reaction with an amine and reduction with sodium cyanoborohydride[9]. Non-aqueous solvents include dimethylsulphoxide (DMSO)[10] and *N*,*N*-dimethylformamide (DMF)/LiCl mixtures (2% w/v)[11]. The former cannot be used with carbodiimides since DMSO brings about facile oxidation of alcohols under these conditions[12,13]. In fact, there is no reference in the literature to the use of carbodiimides for the esterification of dextrans.

This paper reports the esterification of dextran in non-aqueous solution. Solvents other than water are very limited in number; we have successfully used media based on formamide and dimethylsulphoxide and have examined systems proposed by others. Formamide was employed in 1952[14] for the acylation of dextran with acid anhydrides. The synthesis of bioactive esters of (I) and coupling reactions of dipyridamole with dextran are

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included, together with the preparation of ester intermediates suitable for the synthesis of graft copolymers of dextran and vinyl monomers.

EXPERIMENTAL

Materials

Generous samples of the potent inhibitor of platelet aggregation BW 245C (**I**) were gifts from Dr N. Whittaker of the Wellcome Research Laboratories; (**I**) was also available labelled with tritium at positions C-5 and C-6 so that mixtures of convenient radioactivity could be prepared immediately prior to use.

Similar procedures were adopted for model compounds of (**I**). Palmitic acid tritiated at positions C-9 and C-10 (Amersham International) was mixed with the unlabelled species (B.D.H.) previously purified by recrystallization from distilled acetone (M.pt. 63°C). 1-^{14}C sodium butyrate (Amersham International) was acidified with dilute hydrochloric acid before addition to unlabelled butyric acid (B.D.H.) which had been freshly distilled on a vacuum line. 2-bromopropionic acid was used in a similar fashion.

N,N'-Dicyclohexyl carbodiimide (DCC, Fluka) was distilled under diminished pressure (b.p. 130°C/3 mmHg) before use. Some experiments were carried out with *N,N'*-dicyclohexyl (^{14}C) carbodiimide (Amersham International) dissolved in a suitable solvent (usually formamide or DMF) and added to the purified unlabelled species to give stock solutions of convenient activity. Carbonyl di-imidazole (CDI, Fluka) was stored in a desiccator at 0°C over silica gel and used directly.

Dextran of nominal molar mass 71 200 (Sigma Chemical Co.) was dried in a vacuum oven at 50°C for 24 h before use. *p*-Dimethylaminopyridine (DMAP) and *p*-pyrrolidinopyridine (PYP) (both from Aldrich Chemical Co.) were recrystallized from distilled ethyl acetate and stored over silica gel at 0°C.

All reaction solvents were purified by distillation and stored over molecular sieve 4A. Formamide was first allowed to stand over sodium bisulphate and magnesium sulphate while DMF and DMSO were treated with phosphorus pentoxide and calcium sulphate respectively, before being distilled under diminished pressure.

Manganese and rhenium carbonyls ($Mn_2(CO)_{10}$) were purified by sublimation in a vacuum.

Techniques

With DCC as coupling agent, the relative molar proportions of acid, DCC and pyridine-based catalyst were 1.0, 1.1 and 0.1, respectively[15]. The acid was mixed with a solution of dextran and the catalyst before addition of DCC. With CDI, proportions were similar, but the order of addition was modified as described in the text.

After reaction the polymers were purified by multiple reprecipitation. Highly coupled dextrans were precipitated from methanol into water; polymers with low degree of coupling were precipitated from water into methanol. In a few cases with intermediate extents of coupling precipitation was carried out from water into a 60:40 (v/v) mixture of methanol and ethyl acetate.

The optical system used in grafting with $Mn_2(CO)_{10}$ ($\lambda = 436$ nm) or $Re_2(CO)_{10}$ ($\lambda = 365$ nm) has been described previously[3].

Incorporation of labelled residues in the polymers was measured by conventional scintillation counting in solvents consisting of 1 ml methanol + 10 ml KL354 (Koch–Light) or 1 ml distilled water + 10 ml Aquasol II (New England Nuclear), depending on the solubilities of the polymers.

Elemental analyses were carried out by Elemental Microanalysis Limited, Okehampton, Devon.

RESULTS AND DISCUSSION

Sanchez-Cháves and Arranz[11] reported the successful use of the DMF/LiCl system as solvent in the reaction of dextran with n-alkyl chloroformates. However, we have been unable to obtain adequate esterification of carboxylic acids with dextran using this solvent with DCC. Unsatisfactory results were also obtained in media in which one component was insoluble, e.g. dextran does not dissolve in DMF and gives negligible coupling; a similar result was obtained with insoluble acids, e.g. palmitic acid in formamide. We have confirmed that water is unsuitable as a medium for esterification, even with water-soluble carbodiimides such as cyclohexyl-morpholinoethyl carbodiimide. Aqueous mixtures with DMF which dissolve dextran also give poor degrees of coupling.

Couplings with DCC in solvents containing formamide

For a review of DCC chemistry see ref. 16. Methylene chloride is a preferred solvent for esterification with DCC and we found that addition of this, together with DMF, enhanced the extents of coupling in formamide solution. DMF is required to prevent precipitation of dextran by methylene chloride. Our 'standard' solvent F consisted of: $HCONH_2$ 50, DMF 45, CH_2Cl_2 5% v/v.

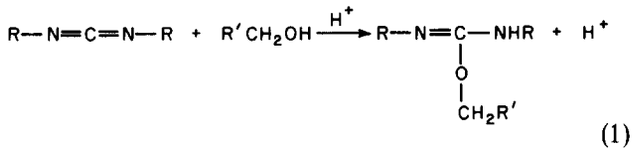
Typical results for coupling of butyric acid as model compound are presented in *Table 1*.

Use of coupling agents for polymer reactions raises special problems since side reactions incorporating unwanted groups in the polymer should be minimised, especially if bioactivity is to be assessed. Corresponding products arising with small molecules can generally be

Table 1 Esterification of dextran with butyric acid in solvents containing formamide. Coupling agent DCC; concentration of dextran 50 g dm^{-3}; reaction time 19 h; temperature 25°C

Butyric acid concentration (g dm^{-3})	% Acid converted	% N in polymer: by analysis	% N in polymer: from labelled DCC	Comments: solvent, reaction time
DMAP catalyst				
50	29.8	0.79		F
50	13.8	0.13	0.03	F
50	6.0	0.16		formamide alone
25	20.0	0.32		F
25	6.0			formamide alone 48 h, −15°C
24	8.5			formamide alone 48 h
24	13.3			48 h
13	3.3	0.15	0.10	F
PYP catalyst				
50	92.5	0.82	0.76	F
50	48.9	0.32	0.14	F

separated by conventional procedures. Numerous side-reactions may occur with carbodiimides as coupling agents in esterification; one such reaction with dextran leads to the formation of an isourea ether[13,16]:



(1)

We therefore thought it desirable to check the nitrogen contents of our polymers, both by elemental analysis and measurement of radioactivity using ^{14}C-labelled DCC. Such a check was also deemed necessary in view of the use of formamide and DMF in the reaction medium. Low nitrogen contents were generally found in the polymers after esterification (*Table 1*). In our experience elemental analyses are not very reliable with these polymers; thus unreacted dextran samples were found to have nitrogen contents up to 0.1% and the carbon percentages were always low. Nitrogen contents arising from DCC determined by scintillation counting appeared to be somewhat less than those determined by microanalysis.

Table 1 shows that in formamide solution the extents of coupling were smaller than those in the preferred reaction medium. It is also clear from *Table 1* that *p*-pyrrolidinopyridine is a better catalyst than *p*-dimethylaminopyridine, although it may lead to slightly higher nitrogen contents in the polymer.

Couplings with CDI

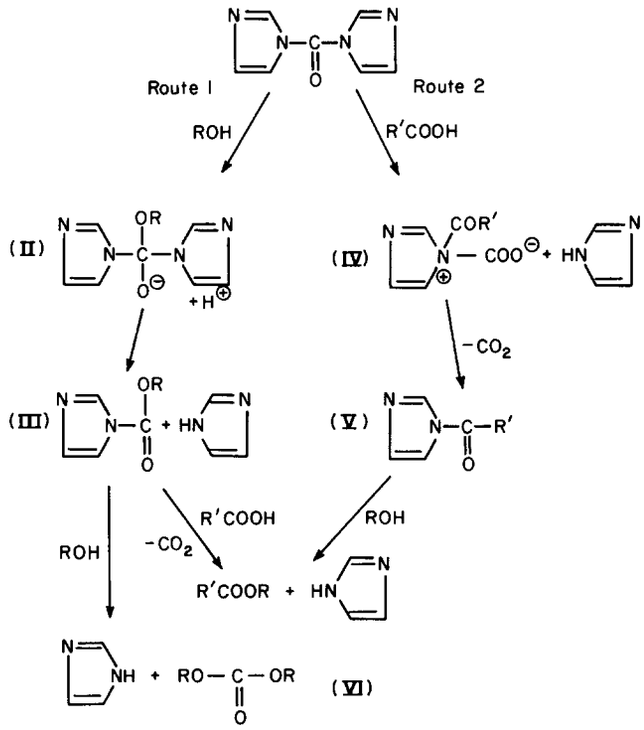
For a review of CDI chemistry see ref. 17. The difficulty of preventing the incorporation of nitrogen-containing groups in dextran on esterification led us to explore the possibilities offered by CDI in these reactions. We have examined both the preferred formamide based system and also DMSO as reaction media.

Results are presented in *Table 2*. Satisfactory couplings may be obtained in DMSO. We were unable to detect any ketone oxidation products from dextran with the aid of 2,4-dinitrophenylhydrazine and the pungent odour of dimethylsulphide did not develop. (When DCC was substituted for CDI both tests gave strongly positive results.) Model experiments with isopropanol instead of dextran behaved identically.

DMSO appears to be the only common solvent for palmitic acid and dextran suitable for coupling by this method, although we obtained only a low conversion.

Carbonyl di-imidazole reacts with alcohols and carboxylic acids[18] to form esters as set out in *Scheme 1*. In principle, ester synthesis may be effected by either route, i.e. starting with the CDI+ROH, or CDI+R'COOH reactions. Both routes 1 and 2 yield the ester R'COOR, via intermediates (**II**), (**III**) and (**IV**), (**V**), respectively; with small molecules as reactants this product may be readily purified by conventional techniques. However, intermediate (**III**) of route 1 enters into further reaction with the alcohol yielding a carbonate (**VI**); with a polyhydroxylic macromolecule such as dextran as reactant this will result in crosslinking, so that the required ester forms part of a network from which it cannot be separated.

Scheme 1



Route 2, therefore, seems more appropriate with these macromolecules and can be followed as a two-stage process. CDI is first reacted with the acid to yield (**IV**) which readily decarboxylates to the acylimidazole (**V**). On addition of the alcohol, (**V**) is converted to the required ester. Note that any unreacted CDI is available for reaction by route 1.

Table 2 Esterification of dextran in dimethyl sulphoxide and 'standard' formamide solvent F. Coupling agent CDI: catalyst PYP; concentration of dextran: 50 g dm^{-3}; temperature 25°C

Butyric acid concentration (g dm^{-3})	% Acid converted	% N in polymer by analysis	Solvent	Conditions
25	14.6	0.51	F	17 h
50	32.0	0.29	F	17 h
50	2.5	0.24	F	2 h
50	1.0	0.21	F	10 min
				2 stage reactions, see text
50	2.8	0.13	F	$t_1=2$ h, $t_2=10$ min
50	24.6	0.4	F	$t_1=2$ h, $t_2=2$ h
50	91.7	0.25	F	$t_1=2$ h, $t_2=17$ h
50	24.5		DMSO	$t_1=2$ h, $t_2=17$ h
50[a]	3.6		DMSO	$t_1=2$ h, $t_2=17$ h, 45°C
13	1.9	0.1	F	$t_1=10$ min, $t_2=17$ h
13	2.4	0.14	F	$t_1=30$ min, $t_2=17$ h

[a] Palmitic acid

We have examined both routes for preparing esters of dextran. Route 2, carried out in two stages, is very effective (see *Table 2*). In *Table 2*, t_1 and t_2 are the dwell times for (1) reaction between CDI and R′COOH and (2) the subsequent reaction with dextran, respectively. A value of $t_1 = 2$h is clearly suitable for butyric acid in solvent F; high couplings are attainable, with low N-contents in the polymer.

In a single-stage reaction, esterification will proceed by both routes. Good degrees of coupling may be obtained in this way but clearly the technique is less efficient than the two-stage method (*Table 2*). The single-stage experiment with a low acid concentration (*Table 2*) gave a rather high N-content. Incorporation of nitrogen may arise from the route 1 intermediate (**III**), so this result probably indicates that only a portion of the CDI had reacted with the acid, the remainder following route 1. A longer first-stage reaction, for a given concentration, may give lower nitrogen contents.

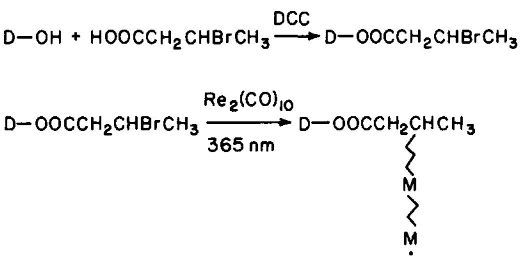
When the reaction is carried out with dextran in the absence of carboxylic acid only route 1 is possible and the final product is (**VI**), i.e. dextran crosslinked by carbonate groups. Intra-chain as well as inter-chain carbonate links may be formed. The infra-red spectrum (KBr disc) of the product (thoroughly dried at 60°C in vacuum for several days) shows strong absorption at $1760\,cm^{-1}$ which is absent from the spectrum of unmodified dextran but present as a characteristic band in the spectra of low-molecular weight alkyl carbonates.

The total number of carbonate crosslinks is determined by the initial ratio [CDI]/[dextran]; as this ratio is increased the product changes from homogeneous solution to a thin gel and then to a rigid crosslinked structure.

A corollary to this work is that 'activation' by CDI of OH groups in columns intended for affinity chromatography[19], e.g. those composed of agarose, must involve the formation of carbonate crosslinks as described above.

Synthesis of graft copolymers of dextran

The esterification technique described permits the introduction of functional groups suitable for the synthesis of graft copolymers of dextran. A procedure we have used to graft vinyl polymers to dextran is indicated in *Scheme 2* in which D–OH and M represent dextran and a vinyl monomer respectively.



Scheme 2

Esterification was carried out with equal concentrations ($50\,g\,dm^{-3}$) of dextran and 2-bromopropionic acid in solvent F using DCC with DMAP as catalyst. The extent of conversion of the acid after 19 h was 41.4%. The ester product contained 15.1% Br in close agreement with the value calculated (15.08%) from the weight increase after reaction.

Table 3 Grafting of 2-bromopropionate ester of dextran (Br 15.1% corresponding to 180 2-bromopropionate residues per chain). 25°C, 0.1 g dextran ester in 5 ml total volume. Monomer concentration 40% v/v in formamide. $[Re_2(CO)_{10}] = 2 \times 10^{-3}\,mol\,dm^{-3}$; $[Mn_2(CO)_{10}] = 2 \times 10^{-3}\,mol\,dm^{-3}$

Monomer	Reaction time (minutes)	% Graft (w/w, on dextran ester)
Methyl methacrylate	180	675
2-Hydroxyethyl methacrylate	45	640
Acrylamide[a]	45	976
Acrylic acid	30	1380

[a] $Mn_2(CO)_{10}$ was used in this experiment

Table 4 Coupling of **I** to dextran (molar mass 71 200.) DCC/DMAP; temperature 25°C; reaction time 19 h

Concentrations ($g\,dm^{-3}$)				
Dextran	(I)	Solvent	Conversion of (I) (%)	Content of (I) in final polymer (% w/w)
12	2	Formamide	0.6	0.1
54	46	F	39	25

Some results on the grafting of different monomers are presented in *Table 3*. The 2-bromopropionate ester is very active as a co-initiator with $Mn_2(CO)_{10}$ and $Re_2(CO)_{10}$. The advantages of this grafting technique have been described elsewhere[20].

Coupling of bioactive agents to dextran

As already stated, we have coupled the potent anti-platelet agent (**I**) to dextran by direct esterification. Coupling with the aid of DCC/DMAP has been carried out under the conditions shown in *Table 4*. By varying the reaction conditions polymers with widely different contents of (**I**) may be prepared. Studies of the inhibition of platelet aggregation by these soluble polymers as a function of their contents of (**I**) and particularly the remarkable synergistic effects obtained on dilution with the parent dextran have been reported[1]. Such dilution produces an increase in the activity of coupled (**I**) estimated on a molar basis which may easily reach two orders of magnitude. These results may be of general significance in the study of interactions of cells with polymers coupled to bioactive agents.

Note that since the antiplatelet activity of (**I**) is critically dependent on the presence of the hydroxyl at C-15, coupling must be carried out with a large excess of dextran-hydroxyl to minimize reaction of the hydroxyl group of (**I**). Use of CDI may not be appropriate for coupling (**I**) to polymers.

If desired, drugs containing hydroxyl groups may easily be coupled via a carbonate link by use of CDI following route 1 (*Scheme 1*). For example, we have converted dipyridamole, which contains four OH groups per molecule without other functionality, into a crosslinked network by use of CDI/PYP or CDI/DMAP in formamide or DMSO. A variety of solvents may be used for this purpose. Coupling of dipyridamole to dextran may also be effected in formamide or solvent F; both soluble adducts and crosslinked gels have been obtained in this way. Other hydroxylic polymers such as poly(ethylene glycol) may also be coupled.

Several polymer adducts synthesized by these reactions are currently undergoing tests for bioactivity.

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