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Synthesis and Inverse Emulsion Polymerization of Aminated Acrylamidodextran

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Abstract—A chemically modified form of dextran was prepared, having a polymerizable moiety (acrylamide) and a reactive functional group (primary amine). Dextran was activated with 4-nitrophenylchloroformate (24 mol per polysaccharide, 9·8 mol per 100 glucose residues); 9·8% glucose residues were converted to aliphatic carbonates and 5·2% were converted to cyclic carbonates. The activated dextran was coupled with trityldiaminoethane (8 mol per 100 glucose residues), reactivated with 4-nitrophenylchloroformate, then coupled with acryloamidodiaminohexane (6·8 mol per 100 glucose residues). The trityl group was removed by hydrolysis with trifluoroacetic acid to yield the required aminated acryloamidodextran. The modified dextran was shown to be polymerizable by inverse emulsion polymerization. Submicron particles were successfully prepared, containing functional amine groups suitable for preparing drug conjugates or for modifying the surface properties of this biomaterial.

The dextrans, natural polysaccharides of various molecular weights, have already been used in medicine as plasma expanders and these water-soluble polymers have been chemically modified so that drugs can be attached to them (Schacht et al 1984, 1985) in an attempt to obtain an extended period of activity for the drugs, reduced toxicity, and specific targeting of tissues. These goals might also be reached by using insoluble forms of the polymers (Edman et al 1980; Artursson et al 1984; De Luca & Rypacek 1988).

When compared with the soluble vectors, the insoluble carriers might provide an effective protection of protein drugs against proteolytic inactivation, avoiding undesirable immune reactions.

The presence of functional groups on the particle surface is essential when a drug has to be attached to the carrier or the surface has to be modified, as stressed by McLeod et al (1988) and Lenaerts et al (1989). This paper describes an original method to modify dextran so that it could bear both acrylamide and primary amine groups.

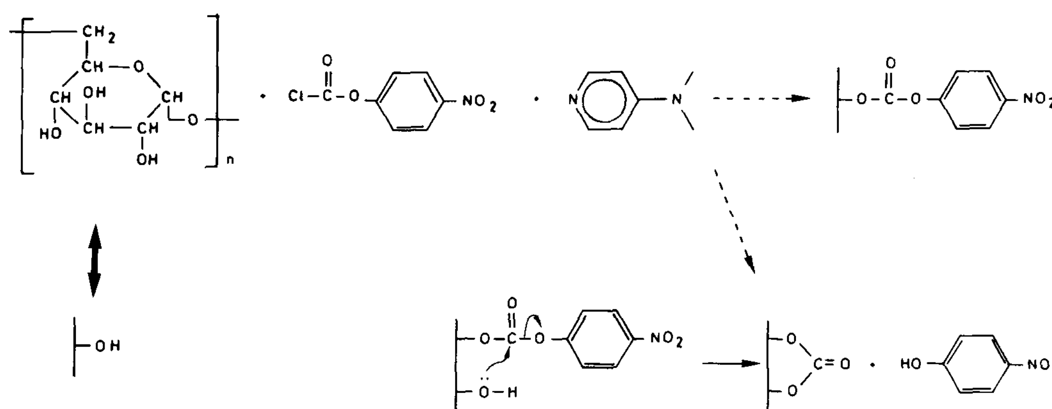
Materials and Methods

The dextran, fraction T40, was provided by Pharmacia (Uppsala, Sweden). 4-Nitrophenylchloroformate, 4-dimethylaminopyridine (DMAP), and *N,N,N',N'*-tetramethylethylene diamine (TEMED) were purchased from Janssen Chimica (Beerse, Belgium). Glycidyl acrylate and *N-t*-butoxylcarbonyl-1,6-diaminohexane were obtained from Fluka AG (Buchs, Germany).

Pluronic F68 was a gift from ICI Belgium NV/SA (Kortenberg, Belgium). All other products were of analytical grade.

Trityldiaminoethane (TTDAE, 1-triphenylmethyl-amino, 2-aminoethane) was synthesized as described by Vandoorne et al (1991). The synthesis of acrylamido-6-aminohexane (AAH) was carried out as reported by Stahl et al (1978). Polysaccharide was reacted with glycidyl acrylate according to the procedure outlined by Edman et al (1980), Artursson et al (1984) and De Luca & Rypacek (1988).

^{1}H NMR spectra were recorded using a WH-Bruker 360



Scheme 1

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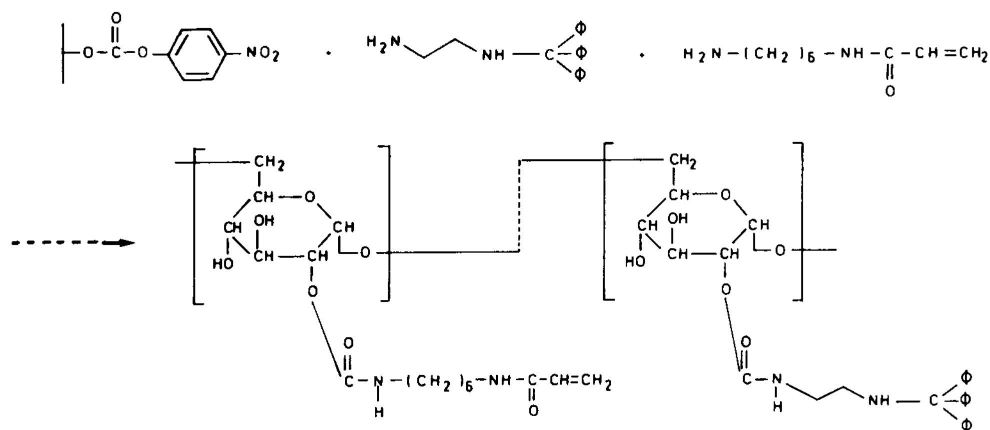
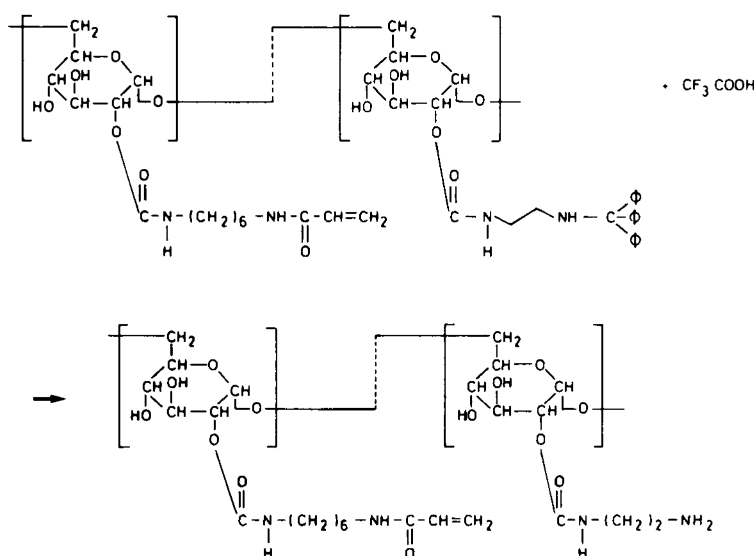


Scheme 2



Scheme 3

MHz apparatus. Preparative size exclusion chromatography was performed on a Pharmacia column (C26/100) filled with Sephadex G25.

4-Nitrophenylchloroformate activation of dextran (Scheme 1)
The procedure was that of Vandoorne et al (1985). Briefly, dextran (2 g, or 12·34 mEq anhydroglucopyranoside) and DMAP (2·5 mg, or 20·5 μmol) in 120 mL dried DMSO/pyridine (1/1, v/v) were added to 4-nitrophenylchloroformate (1·72 g or 8·5 mmol) under stirring at 0°C. After 4 h at 0°C and under a nitrogen atmosphere, the activated dextran was slowly precipitated into anhydrous ethanol (50 mL), washed 5 times and finally dried under vacuum. Contents of 4-nitrophenylcarbonate and total carbonate groups were measured as detailed by Vandoorne et al (1985).

Sequential coupling of trityldiaminoethane and AAH to preactivated dextran (Scheme 2)
Trityldiaminoethane (64 mg or 213 μmol) was added under stirring to the pre-activated dextran (0·5 g) previously dissolved in a dried DMSO/pyridine mixture (1/1, v/v). After 48 h at room temperature (21°C), AAH (44 mg or 213 μmol) was added to the reaction mixture and the reaction continued for 24 h. The polysaccharide was isolated by precipitation in cold ethanol. The collected white precipitate was washed 5 times with ethanol (50 mL) and dried under vacuum.

Deprotection of the tritylamino groups (Scheme 3)
The trityl-nitrogen bond, which is very sensitive to acidic media, was readily hydrolysed by trifluoroacetic acid (0·2 mL) added under stirring to an aqueous solution (4%) of the modified dextran. After 24 h at 24°C, the reaction mixture was filtered, and the filtrate neutralized at pH 7·0 with sodium hydroxide. The aminated acrylamidodextran was further purified by preparative gel permeation chromatography.

Copolymerization of the aminated acrylamidodextran
Pure aminated acrylamidodextran was dissolved in a sodium phosphate buffer (5 mM, pH 7·0), and *N*,*N*′-methylenebis-

acrylamide (BIS) added. After addition of ammonium persulphate (0·08 M), the aqueous phase was dispersed with an ultra-turrax homogenizer at maximum speed in a chloroform/toluene mixture (1/4, v/v) under a nitrogen atmosphere. The emulsion was stabilized with Pluronic F68 previously dissolved in the organic phase (0·25%, w/v). The inverse emulsion polymerization was initiated by the addition of TEMED (1·7 mM) and after 30 min at room temperature, surfactant was removed from the polymerization medium by repeated centrifugation of the latex at 40000 *g*.

Characterization of the particles

The size of the purified latex was determined by photon correlation spectroscopy analysis with a Coulter N4 MD (Coulter Electronics Ltd, Luton, UK). The autocorrelation functions were analysed by least-square fits.

The morphology and the size of the particles were also determined by scanning electron microscopy (JEOL, JSM-840 A) after Au-Pd sputtering (Balzers, SCP-20).

The unreacted amines (recovered by centrifugation of the latex) and those immobilized on the latex were measured by visible spectrometry after reaction with trinitrobenzene sulphonic acid as reported by Snyder & Sobocinski (1975).

Results and Discussion

Chemistry

The percentage of glucose residue converted into aliphatic and cyclic carbonates respectively, was measured according to Vandoorne et al (1985) and is reported in Table 1.

One vinyl group per 10 to 20 glucose residues has been recommended to polymerize dextran into a three-dimensional network (Edman et al 1980; De Luca & Rypacek 1988).

As measured by ^{1}H NMR (Table 2), the reaction of TTDAE is almost quantitative. The measurement relies upon the integration of signals at 4·5 ppm (anomeric protons) and 7·1 to 7·4 ppm (aromatic protons).

^{1}H NMR also showed that the coupling of AAH to dextran had failed. This failure is more likely due to the nearly quantitative consumption of the aliphatic carbonates by TTDAE and the lower reactivity of the cyclic carbonates. Therefore, a second activation was performed; 7·5% of glucose residues were then activated and reacted with AAH. Fig. 1 shows the presence of AAH on the polysaccharide. According to the integration of signals at 1·25 ppm (protons c of AAH) and at 4·55 ppm (anomeric protons of glucose residues), 6·8% of the glucose residues were added corresponding to a 90·7% coupling (Table 2).

The infrared spectrum of the functionalized dextran (Fig. 2) shows the complete global disappearance of the cyclic and aliphatic carbonate group at 1805 and 1765 cm^{-1}, respectively.

Table 2. Sequential coupling of trityldiaminoethane and acrylamidodiaminohexane to activated dextran.

Functionalized dextran from 0·5 g activated dextran	0·37 g
Trityldiaminoethane coupled per 100 glucose residues	8
Acrylamidodiaminohexane coupled per 100 glucose residues	0*

*After a second activation of the coupled dextran, 6·8 mol acrylamidodiaminohexane was coupled per 100 glucose residues.

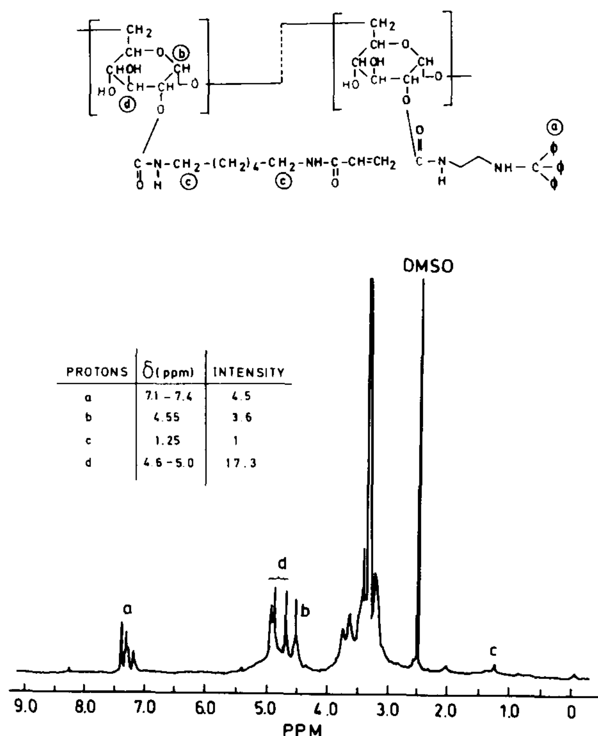


FIG. 1. ^{1}H NMR spectrum after the reaction of chloroformate-activated dextran with trityldiaminoethane and acrylamido-6-aminohexane. Solvent: $(CD_3)_2SO$.

The tritylamino groups attached to dextran were hydrolysed by trifluoroacetic acid to release the parent primary amines. The final product was purified by size exclusion chromatography (Sephadex G25).

As shown by ^{1}H NMR (Fig. 3), the deprotection was mainly quantitative, since signals of the trityl protons at ≈7·2 ppm were virtually absent. The presence of primary amino groups was confirmed by titration with trinitrobenzene sulphonic acid (TNBS).

The inverse emulsion polymerization of the acrylamidodextran was carried out to prepare submicron particles (Edman et al 1980).

Table 1. Activated dextran from reaction with 4-nitrophenylchloroformate.

Yield from 2·2 g dextran	2·21 g
4-Nitrophenylchloroformate converted	24 mol per polysaccharide 9·8 mol per 100 glucose residues
Glucose residues converted to aliphatic carbonate	9·8%
Glucose residues converted to cyclic carbonate	5·2%

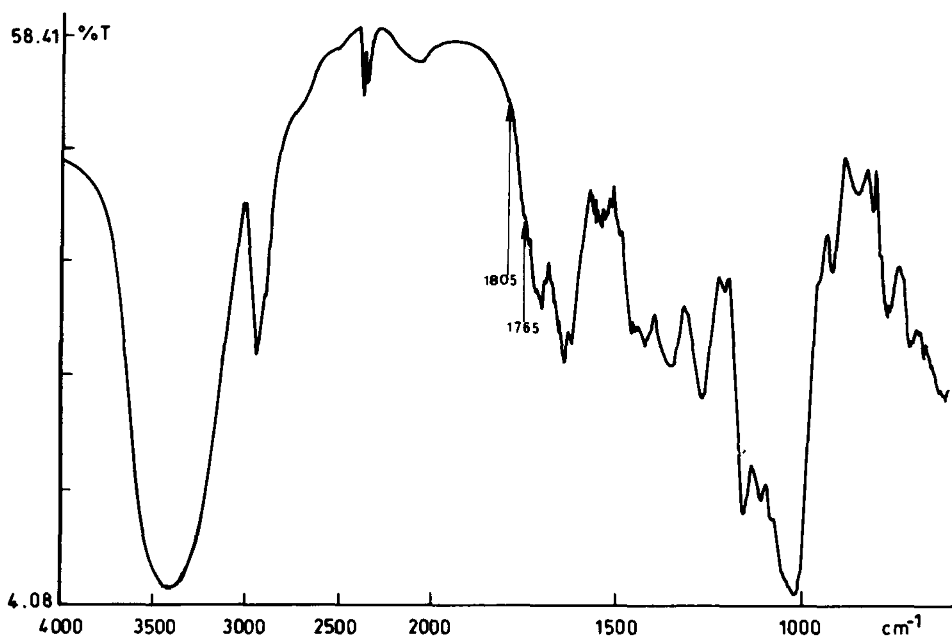


FIG. 2. IR spectrum of the activated dextran after reaction with trityldiaminoethane and acrylamide 6-aminohexane.

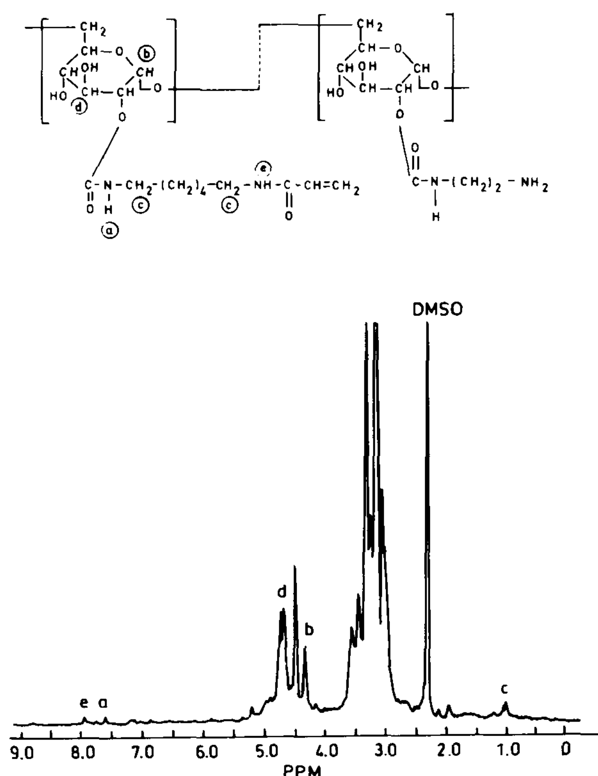


FIG. 3. ^{1}H NMR spectrum of dextran bearing acrylamido and deprotected primary amino groups. Solvent: $(CD_3)_2SO$.

The emulsion polymerization was initiated by a redox system: ammonium persulphate-TEMED, and conducted in the presence or absence of various concentrations of a cross-linking agent: *N,N'*-methylenebisacrylamide. Table 3 reports characteristics of the latex prepared in the presence of the aminated acrylamidodextran compared with those of the acryloyldextran prepared according to the procedures of Edman et al (1980) and De Luca & Rypacek (1988). Even in the absence of a difunctional co-monomer, a hydrophilic latex is formed, the degree of cross-linking of which is large enough to withstand solubilization during the purification procedure. It is clear that the aminated acrylamidodextran can be polymerized with formation of nanoparticles in spite of the low percentage of vinyl units.

The distribution size of the latex was measured by photon correlation spectroscopy (Table 3, Fig. 4) and scanning electron microscopy (Fig. 5). Although our latex was purified by repeated centrifugation-resuspension cycles, it is very difficult to eliminate surfactant traces, which leave a smooth coat over the particle surfaces and inhibit clear observation of the materials (Kreuter 1983).

Nevertheless, the formation of submicron particles is convincingly supported. In agreement with the literature, there is a broad size distribution of the latex produced by inverse emulsion polymerization (Graillat et al 1986; Glukhikh et al 1987). The large agglomerates present may be aggregates or may arise from large particles in the original emulsion.

Photon correlation spectroscopy shows a bimodal distribution (Fig. 4). The initiation takes place both in the monomer droplets and in the micelles. As suggested by Arshady (1988), a bimodal system results from simultaneous suspension and emulsion polymerization mechanisms. Furthermore, the particle size distribution seems to be affected by the nature of the unsaturated groups grafted onto the polysaccharide. The size distribution of the polyacryldextran particles is centered around 460 nm, whereas that of polyacrylamidodextran is more dispersed and larger than 500 nm. It might be suggested that the amino groups attached to the acrylamidodextran and protonated at the pH

Table 3. Inverse emulsion polymerization of acryloyldextran and aminated acrylamidodextran.

Monomer concn in dispersed aqueous phase (D-T-C ratio[a]) Dextran	Toluene	Cross-linker	Recovery of primary amines (%)	Size (nm)	Particle size analysis Intensity (%)	Size (nm)	Intensity (%)
acryloyldextran							
10	0·225	—		92	15	460	85
10	0·281	620		80	8	460	92
10	0·375	40		35	3	462	97
10	0·750	70		184	10	562	90
Aminated acrylamidodextran							
10	0·213	—	42·7	145	10	788	90
10	0·266	0·20	37·2	295	30	1224	20
10	0·710	70	47	96	10	580	90

[a] As defined by Edman et al (1980).

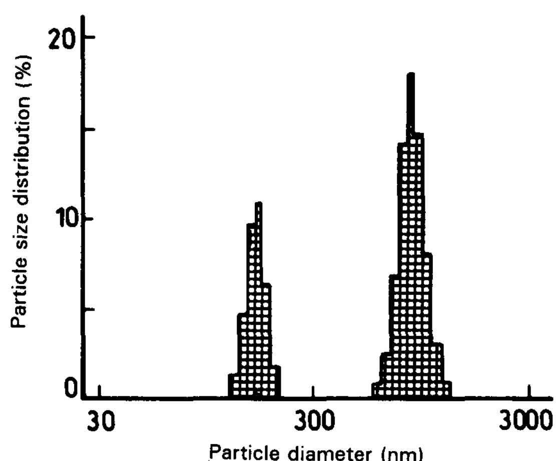


FIG. 4. Size distribution of the aminated acrylamidodextran particles as determined by photon correlation spectroscopy (Coulter N4-MD). Diluent: water.

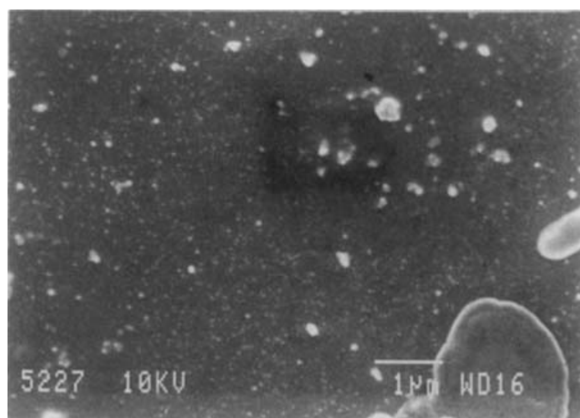


FIG. 5. Scanning electron micrograph of aminated acrylamidodextran particles.

of the emulsion polymerization, affect the interfacial properties of the polysaccharide and accordingly, the stability of the inverse emulsion.

The reaction of TNBS with the aminated acrylamidodextran particles (Table 3) supports the availability of primary amine groups on the particle surface. These groups should be useful to modify the latex surface.

The recovery of the amino groups after polymerization is far from quantitative: 37–47% of the amino groups of the acrylaminodextran are associated with the latex surface. Since after polymerization, the supernatant solution recovered by centrifugation at 40000 *g* contains 20% of the original amines, it is clear that the reticulation of the acrylamidodextran is not quantitative. It is assumed that non-detected amines (33–47% of the total) are buried inside the cross-linked dextran particles. The possibility that the primary amine groups grafted onto the dextran would react with the vinylic unsaturations was rejected, as we have verified in separate experiments that AAH does not react with acrylamide monomers in the polymerization conditions used.

To be usable, particulate drug carriers must have properties of biocompatability and biodegradability as well as the ability to bind with various drugs and to release them in a way which can be closely controlled. Natural polymers are candidates since they can form particles and can produce functional groups internally or on the surface of the particles. These groups can bind drugs, and can also modify the surface properties of the carrier which is particularly important in order to regulate biocompatibility as stressed by Hastings (1985), distribution in the body and clearance after absorption of nanoparticles as discussed by Illum et al (1989) and Laakso et al (1986).

Baillie et al (1987) have reported the preparation of polyacryl starch microparticles functionalized with amino groups in order to bind an antileishmanial drug. Their procedure involves the reticulation of acryloylated starch in the presence of AAH in an inverse emulsion. Adopting a similar procedure with acryloylated dextran, we have verified that AAH does not favourably react with the acrylic derivative of dextran. Our observations can be related to the different repartition behaviour of the two species in the inverse emulsion. We have indeed verified that AAH was partially re-extracted in the organic phase of a toluene/chloroform/water mixture, whereas the modified dextran is soluble only in the aqueous phase. The present work avoids such difficulties, modifying the polysaccharide before polymerization.

The chloroformate activation method of Vandoorne et al

(1985) allowed dextran to be sequentially combined with a monoprotected diamine and an acrylamido derivative; 8 and 6·8% of the glucose residues were reacted with amino and acrylamido groups, respectively. Although the activation of dextran was carried out in such a way that 20% of the glucose residues were activated, only 8% of these residues reacted with TTDAE in contrast with AAH which was added in a second step and remained unreacted. This experimental result was accounted for by the partial rearrangement of the 4-nitrophenyl carbonate groups into the less reactive cyclic carbonate moieties. Thus coupling of AAH to dextran required a second activation of the polysaccharide with chloroformate. It is clear that the activation of the glucose residues is still to be optimized and that experimental conditions under which TTDAE and AAH could be simultaneously combined with dextran are a valuable target.

The polymerizability of the aminated acrylamidodextran was tested in the presence and absence of a difunctional comonomer, *N,N'*-methylenebisacrylamide. In every case (Table 3), an insoluble hydrophilic latex was formed, which showed a bimodal particle size distribution as might be anticipated from the experimental polymerization conditions. On the basis of the content in acrylamido groups (6·8% with respect to the glucose residue) and assuming a complete conversion of these groups, an average number molecular weight of 2500 between cross-links can be calculated in the absence of *N,N'*-methylenebisacrylamide. As a result, the nanoparticles should be degraded in-vivo into soluble low molecular weight products.

The presence of amino groups attached to the nanoparticles allows positive charges (protonated amines) to be combined with the negative charges generated by the initiator SO_4^{2-} groups and to provide ion-exchange properties. This ionic environment could control the kinetics of drug release, and the amino group could be used to bind a drug or a drug-conjugate as well as to modify surface properties of the biomaterial.

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