Carbohydrate Polymers 95 (2013) 569–577

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Aminoalkyl functionalization of dextran for coupling of bioactive molecules and nanostructure formation

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ARTICLE INFO

Article history:
Received 17 November 2012
Received in revised form 15 February 2013
Accepted 3 March 2013
Available online 13 March 2013

Keywords:
Glucan functionalization
Amino alkyl dextran
Immobilization of active molecules
Nanostructure formation
Magnetic nanoparticle

ABSTRACT

Aminopropyl dextrans and mixed aminopropyl cyanoethyl dextrans were prepared from cyanoethyl precursors by full or partial reduction with $CoCl_2/NaBH_4$. Coupling of various aldehydes to the glucan backbone by reductive amination was accomplished with 4-hydroxy-3-methoxybenzaldehyde (vanillin), 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde (BHT-CHO), maltose and maltotriose, and picoline borane as reducing agent. Successful coupling of these representatives for aroma compounds, antioxidants and sugar side-chains were verified by ESI-MS after hydrolysis and by 1D and 2D NMR spectroscopy. Degree of conversion (molar ratio of coupled aldehydes) was estimated from ^{1}H NMR spectra. Formation of secondary and tertiary amines was detectd by ESI-MS. Applying a solvent exchange process, new nanoparticles based on these modified dextrans were prepared with and without addition of iron oxide nanoparticles.

1. Introduction

Amino groups are versatile functions for immobilization of bioactive or other molecules by coupling them to surfaces or polymer backbones. Using various amino functions in molecules and macromolecules, Michael addition, addition to oxiranes or thiocyanates, amide formation and reaction with aldehydes with or without subsequent reduction are widely applied (Hermanson, 2008). In the field of polysaccharides, chitosan obtained from chitin by *N*-deacetylation has been used for such *N*-selective coupling reactions. However, the lack of a spacer between the 2-amino-2-deoxy-glucosyl units of the β-1,4-linked glycan chain restricts applicability. Periodate oxidation of dextran followed by reductive amination has been used by Piehler et al. for the functionalization of silica surfaces for direct immunoprobes (Piehler, Brecht, Geckeler, & Gauglitz, 1996).

The objective of our work with dextran, an α-1,6-linked, branched glucan, is to perform aminofunctionalization while preserving the polysaccharide backbone and maintaining the intrinsic properties of the polymer. To introduce amino functions in a polysaccharide by a polymer analog modification, different approaches have been applied. Fujita et al. described the synthesis of aminoalkyl pullulans by reaction with aminoepoxyalkyl derivatives, e.g. 3-amino-1,2-epoxypropane. Product characterization was only performed by elemental analysis (Fujita, Fukami, & Fujimoto, 1979). However, direct introduction of unprotected amino groups is affected by the higher nucleophilicity of amino groups compared to hydroxyl groups, thus favoring tandem reactions and complex products. This can be avoided by using *N*-protected reagents or by introducing nitrogen in a higher oxidation state. Gonera et al. reported on the Williamson-type *O*-alkylation with phthalimido-protected bromopropylamine and subsequent mild deprotection by reductive hydrolysis (Gonera, Goclik, Baum, & Mischnick, 2002). Cyanoethylation with subsequent reduction to aminopropyl ethers has been widely used and for example applied to inulin (Verraest, da Silva, Peters, & van Bekkum, 1996; Verraest, Zitha-Bovens, Peters, & van Bekkum, 1998) as well as to cellulose and starch (Volkert, Wagenknecht, & Mai, 2010; Gonera, 2004; Gonera et al., 2002). Daly and Munir applied borane-dimethylsulfide in THF for the reduction of cyanoethyl cellulose and proposed an application as ion-exchange resin (Daly & Munir, 1984). Amino functions have also been introduced by click reaction of aminoalkyl azides to pentynyl dextrans, allowing variation of spacer length (Tahir, Lämmerhardt, & Mischnick, 2012). By nucleophilic substitution of 6-*O*-tosyl cellulose with an excess of diamino compounds, *N*-aminoalkyl-6-amino-6-deoxy celluloses have been obtained and used for enzyme immobilization and coating of silica surfaces (Berlin, Klemm, Tiller, & Rieseler, 2000).

Aminopolysaccharides can be used as adsorbent of heavy metal ions (Nakamura, Amano, Saegusa, & Sato, 1992). *O*-Aminoethyl inulin was prepared from inulin with ethylene imine resulting in a maximum DS of 0.76, and the antioxidant effect of

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http://dx.doi.org/10.1016/j.carbpol.2013.03.006

inulin and its derivatives was tested. Modified inulin showed a moderate hydroxyl radical scavenging ability and considerable superoxide-radical scavenging activity (Ren, Liu, Dong, & Guo, 2011). Furthermore, aminoethyl glucans can be applied as components in glycan arrays to analyze carbohydrate–protein interactions. (2-Aminoethyl)-aniline has been coupled to the reducing end, and the so modified carbohydrate directly been fixed by amide formation on a *N*-hydroxy-succinimide (NHS)-activated carboxyl groups bearing glass surface (Seo, Kim, Hwang, & Cha, 2010). By such coupling reactions on surfaces, aminoethyl glucans act as useful interfaces for glycoconjugation (Sardzik et al., 2010). Aminopropyl amylose coatings on PVDF surfaces have been shown to be inert against unspecific protein absorption (Ademovic, Gonera, Mischnick, & Klee, 2006). They have also been successfully applied for the stabilization of horseradish peroxidase (Gonera, Mischnick, & Ukeda, 2004). Polymers with pending amino groups like polyethylene imine have been decorated with maltooligosaccharides by reductive amination to investigate their ability as DNA/siRNA transporter (Höbel et al., 2011). In the following we present the preparation of aminopropyl dextrans (APD) from cyanoethyl dextrans in a wide range of DS. Self-assembly of fully and partially reduced cyanoethyl dextrans and coupling of some model aldehyde compounds as vanillin (Van), 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde (BHT-CHO), maltose (G_2), and maltotriose (G_3) was achieved by reductive amination.

2. Experimental

2.1. Materials

Dextran from *Leuconostoc ssp.* (M_w 6 kDa, puriss) was purchased from Fluka. Cobalt(II)-chloride (p.a.) was received from Across Organics. 2-Picoline borane complex (95%), vanillin (99%) and 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde hemihydrate (99%) were purchased from Aldrich. Maltose monohydrate and $NaBH_4$ were obtained from Merck, maltotriose from TCI Europe N. V. Solvents were of p.a. quality and distilled water was used. Dialysis tubes were obtained from Spectra/Por®, Spectrum Laboratories Inc. (MWCO: 3.5 kDa).

2.2. Instrumentation

^{1}H NMR spectra were recorded on a Bruker AMX 300 spectrometer or a Bruker AMX 400 MHz Avance spectrometer at room temperature (around 5–10 mg sample in MeOH, D_2O or DMSO-d_6). Chemical shifts are given in ppm referred to residual solvent signals. 2D experiments were performed on a Bruker Avance DMX 600 MHz spectrometer (atom number assignment according to Fig. 1).

An Esquire HCT Ultra ETD II (Bruker Daltonics, Bremen, Germany) equipped with an ion trap was used to record ESI-MS spectra (positive mode). Sample solutions (20 μg/mL) were infused directly by a syringe pump with a flow rate of 200 μL/h. Nitrogen was used as drying gas (300 °C, 6 L/min) and as nebulizer gas (10 psi). The following voltages were applied: capillary ± 4500 V, end plate offset ± 500 V, capillary exit ± 100 V, trap drive 45 or 55, smart target 100,000. The maximum accumulation time was adjusted with 200 ms and 50 scans.

The hydrodynamic particle size was analyzed using a Zetasizer Nano ZS (Malvern Instruments Ltd.). DLS measurements were directly performed after dialysis. Nanoparticle dispersions were diluted with distilled water to a final concentration of 1.33 mg PS/mL.

Transmission electron micrographs were obtained using an EF-TEM Libra 120 plus Zeiss microscope operated at 120 kV. Samples were adsorbed to a hydrophilized carbon film, which was supported by a Cu grid (carbon only, copper 300 square mesh) and dried at room temperature. All images were recorded with a 2 × 2k SharpEye cooled CCD camera (Tröndle, Moorenweiss, Germany) in the magnification range from 4000× to 25,000× in the elastic bright-field mode, with the energy slit set to 10 eV.

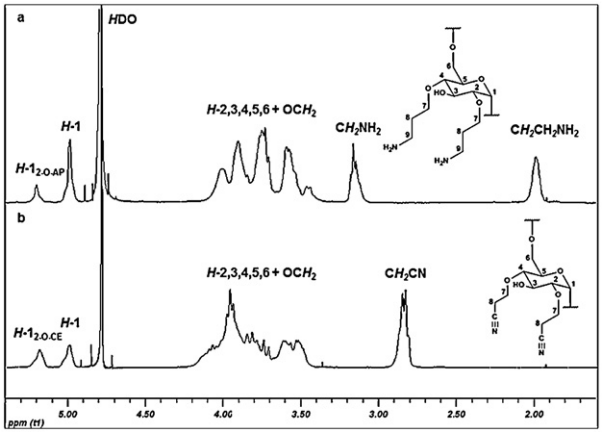

Fig. 1. ^{1}H NMR-spectra (D_2O, 400 MHz) of (a) APD (DS_{AP} = 0.64, D_{red} = 100%) and (b) CED (DS_{CE} = 1.01). The inserted structures show one example of possible substitution patterns.

2.3. Polysaccharide modification

2.3.1. Reduction of cyanoethyldextrans

Cyanoethyl dextrans were prepared by Michael addition using acrylonitrile as has been described (Fiege, Lünsdorf, Atarijabarzadeh, & Mischnick, 2012). Reduction of cyanoethyl dextrans to aminopropyl derivatives was performed according to procedures reported earlier (Gonera, 2004; Gonera et al., 2002; Verraest, da Silva et al., 1996; Verraest, Zitha-Bovens et al., 1998). The cyanoethyl dextrans were mixed with $CoCl_2$ in a big flask and dissolved in water under stirring. Highly substituted cyanoethyl dextrans (DS_{CE} > 2) were first dissolved in 1 mL DMSO and diluted with aqueous $CoCl_2$-solution. Aqueous $NaBH_4$ was added dropwise through a septum to the reaction mixture. Evolving hydrogen was effused through a needle. After 1 h stirring at room temperature, diluted HCl (0.1–1.0 M) was added to dissolve the black precipitate (cobalt boride). Products were isolated by a dialysis against distilled water. Detailed reaction parameters are given in Table 1.

IR (diamant-ATR): $\tilde{\nu}(cm^{-1}) = 3490 - 3234$ (OH + NH), 2919, 2882 (CH, CH_2, aliph.), 1640 (OH), 1550 (NH), 1159, 1099, 1001 (s, C-O).

^{1}H NMR (D_2O, 300 MHz) δ (ppm): 5.21 (1 H, *H*-1, substituted in position 2), 4.97 (1 H, *H*-1, unsubstituted), 4.25 – 3.35 (6 H + DS * 2 H, *H*-2,3,4,5,6a,b,7), 3.15 (2 H, *H*-9), 2.00 (2 H, *H*-8).

2.3.2. Reductive amination

Coupling of aldehydes to the amino functionalized dextran by reductive amination was performed according to literature (Jagadish, Divyashree, Viswanath, Srinivas, & Raj, 2012; Sato, Sato, Okubo, & Yamazaki, 1998; Unterieser & Mischnick, 2011). Detailed reaction parameters are listed in Table 2. Aminopropyldextrans were dissolved or dispersed in methanol under stirring and the aldehyde (4-hydroxy-3-methoxybenzaldehyde, 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde hemihydrate, maltose or maltotriose, 0.8 – 12 eq/NH_2, see Table 2) was added. The pH was adjusted by addition of HCl or NaOH (0.1–1 M). Reaction with aromatic aldehydes was performed at pH 4 (exception: Van-7 at pH 8). Reactions with maltose were carried out at pH 3, 7 and 9, maltotriose was applied

Table 1
Conditions and results of reduction of cyanoethyl dextrans (6 kDa) with cobalt chloride/sodium borohydride in water at room temperature, 1 h reaction time.

Sample	Educt		Reaction parameter			Product			
	DS_{CE}[a]	Weight-in [mg]	$CoCl_2$ eq/CN	$NaBH_4$ eq/NH_2	H_2O [mL]	DS_{AP}[b] residual	DS_{CE}[a]	D_{red}[d] [%]	Weight-out [mg]
APD-1[f]	0.61	200	4.43	15.00	16	0.37–0.47	0.00	100	135–225
APD-2	0.61	300	4.43	15.00	16	0.46	0.00	100	276
APD-3	1.01	200	4.43	15.00	16	0.64	0.00	100	173
APD-4[g]	1.40	400	4.43	15.00	16	0.49–0.63	0.85–0.91	52–60	269–323
APD-5	1.40	300	4.43	15.00	16	1.31	0.00	100	191
APD-6	1.60	200	4.43	15.00	16	1.52	0.04	99	239
APD-7	1.77	150	4.43	15.00	16	1.70	0.00	100	178
APD-8[e,f]	2.20	80	0.55	1.88	9	0.43–0.44	2.31–2.35	15–16	58–68

[a] Calculated from ^{1}H NMR according to Eq. (2).
[b] Calculated from ^{1}H NMR according to Eq. (1) or (3).
[d] Calculated from ^{1}H NMR according to Eq. (4).
[e] Cyanoethyl dextran dissolved in 1 mL DMSO before reaction; DMSO-d_6 was used for NMR experiments.
[f] Reduction performed in duplicates.
[g] Reduction performed in triplicates.

Table 2
Reductive amination of APD with various aldehydes (see Fig. 2), reaction time with aldehyde 3 h, stirred at 45 °C (exception BHT-6, 24 h) and 1.5 h incubation time with picoline borane (exception BHT-6, 29 h), picoline borane was added in 2 portions.

Sample	Educt		Weight -in [mg]	Aldehyde		MeOH [mL]	pH	Picoline borane		MeOH [mL]	D_{conv}[a] [%]	DS_{RCHO}
	DS_{CE}	DS_{AP}		[eq/NH_2]	[mg]			[eq/NH_2]	[mg]			
BHT-5	0.00	1.31	60	10.0	778	2	4	8	710	2	12	0.15
BHT-6	0.00	1.31	40	2.0	104	2	4	16	95	2	53	0.70
Van-2	0.00	0.64	30	4.0	53	3	4	4	83	2	53	0.34
Van-3	0.00	0.64	30	8.0	118	3	4	2	165	4	58	0.37
Van-7	0.00	0.47	30	2.0	23	6	8	1.6	32	4	5	0.02
Van-11	0.04	1.52	20	1.0	19	1	4	20	26	1	33	0.50
Van-12	0.04	1.52	20	0.8	15	1	4	20	21	2	34	0.51
Van-13	0.00	0.47	30	10.0	114	2	4	24	160	1	103	0.48
Van-14	0.85	0.63	25	10.0	121	2	4	12	170	1	77	0.48
Van-15	0.91	0.49	25	12.0	118	2	4	8	165	1	92	0.45
Van-16	2.31	0.44	45	6.0	65	3	4	8	91	1	43	0.19
G_2-1	0.00	0.46	20	4.0	67	3	3	8	42	2	15	0.07
G_2-2	0.00	0.46	20	4.0	67	3	7	8	42	2	18	0.08
G_2-3	0.00	0.46	20	4.0	67	3	9	8	42	2	78	0.36
G_2-4	0.00	0.64	30	4.0								
132	3	9	8	83	2	58	0.37					
G_2-5	0.00	1.70	30	4.0	270	3	9	8	168	2	47	0.81
G_2-6	0.04	1.52	30	4.0	251	3	9	8	157	2	46	0.70
G_2-7	0.00	1.70	30	4.0	270	3	9	8	168	2	70	1.19
G_2-8	0.04	1.52	30	4.0	251	3	9	8	157	2	59	0.90
G_3-1	0.00	0.37	25	4.0	102	3	9	16	43	2	45	0.17

[a] Degree of conversion, see Eq. (5).

at pH 9. The reaction mixture was stirred at 45 °C for 3 h. Picoline borane was added in a molar ratio of 2:1 (referred to aldehyde) in 2 portions. Duration of each reaction step was 45 min at 40 °C. Products were purified by dialysis. Sample BHT-6 was prepared with prolonged reaction times (24 h). Picoline borane was added in two steps (second addition after 5 h). After a reaction time of 29 h with picoline borane, the reaction was stopped by evaporating the solvents and the residue was dissolved in petrol ether. From this solution the product was isolated by precipitation with water, followed by freeze drying.

2.3.2.1. N-(4-Hydroxy-3-methoxybenzyl-3-aminopropyl dextran (Van-13). IR (diamant-ATR): (cm^{-1}) = 3377 (O—H), 3225 (N—H prim., sec.), 2929 + 2887 (CH, CH_2 aliph.), 1662 (N—H prim., sec.), 1519 (aromatic), 858, 817, 787 (=C—H aromatic).

^{13}C NMR (MeOH-d_4, 100.6 MHz) δ (ppm) = 28.00 (*C*-8), 47.35 (*C*-9), 52.70 (*C*-10), 58.85 (*C*-17), 67.80 (*C*-6a,b), 70.24 (*C*-7), 71.87 (*C*-5), 72.12 (*C*-4), 73.56 (*C*-2), 75.38 (*C*-3), 99.90 (*C*-1), 114.40 (*C*-16), 116.67 (*C*-13), 124.40 (*C*-12), 131.23 (*C*-11), 148.31 (*C*-14), 149.45 (*C*-15).

^{1}H NMR (Van-13) (MeOH-d_4, 400 MHz) δ (ppm) = 7.12 (1 H, *H*-16), 6.96 (1 H, *H*-12), 6.88 (1 H, *H*-13), 4.92 (1 H, *H*-1), 4.08 (2 H, *H*-10), 3.99 (1 H, *H*-6), 3.98 (3 H, *H*-17), 3.90 (1 H, *H*-5), 3.78 (1 H, *H*-6), 3.73 (2 H, *H*-7), 3.72 (1 H, *H*-3), 3.50 (1 H, *H*-2), 3.48 (1 H, *H*-4), 3.05 (2 H, *H*-9), 2.01 (2 H, *H*-8).

2.3.2.2. (N-(3,5-Di-tert-butyl-4-hydroxy-benzyl)-3-aminopropyl dextran (BHT-5). IR (diamant-ATR): (cm^{-1}) = 3425 – 3393 (O—H, N—H), 2954 + 2867 (CH, CH_2 aliph.), (N—H prim., sec.), 900 – 800 + 676 (=C—H tetrasubstituted aromatic).

^{1}H NMR (D_2O, 400 MHz) δ (ppm) = 7.33 (2 H, *H*12 + 16), 5.17 (1 H, *H*-$1_{2\text{-}O\text{-}subs}$), 4.96 (1 H, *H*-1), 4.25 – 3.31 (6 H + DS * 2 H, *H*-2,3,4,5,6a,b + *H*-7), 3.14 (2 H, *H*-9), 2.00 (2 H, *H*-8), 1.40 (18 H, *H*-18).

2.3.2.3. Product of reductive amination of APD with maltose (G_2-7). IR (diamant-ATR) (cm^{-1}) = 3293 (O—H), 3229 (N—H prim., sec.), 2929 + 2887 (CH, CH_2 aliph.), 1650 – 1550 1662 (O—H, N—H prim., sec.), 1004 (C—O).

^{1}H NMR: (D_2O, 400 MHz), δ (ppm): 5.45 – 4.80 (1*H* substituted and unsubstituted, 5.11 = *H*-1″), 4.22 – 3.65 (19 H + DS * 2 H, *H*-2,3,4,5,6a,b, *H*-3′,5′,6′a,b *H*-2″,3″,4″,5″,6″a,b + *H*-7), 3.61 (1 H, *H*-2′), 3.46 (1 H, *H*-4′), 3.11 (3 H, *H*-9, *H*-1′), 2.01 (2 H, *H*-8).

^{13}C NMR: (D_2O, 100 MHz), δ (ppm): 107.80 (1 C, *C*-1″), 103.41 (1 C, *C*-1), 84.49 (1 C, *C*-4) 84.44 (1 C, *C*-4′), 82.15(1 C, *C*-5″), 75.90

– 72.22 (10 C, *C*-5, *C*-3″, *C*-2″, *C*-3, *C*-2′, *C*-2, *C*-3′, *C*-4″, *C*-5′, *C*-7), 65.61 – 63.25 (*C*-6′, *C*-6, *C*-6″), 53.47 (1C, *C*-1′), 48.93 – 48.56 (1 C, *C*-9), 29.81 – 29.76 (1 C, *C*-8).

2.3.2.4. Product of reductive amination of APD with maltotriose (G_3). ^{1}H NMR: (D_2O, 400 MHz), δ (ppm): 5.41 – 4.80 (1 H substituted and unsubstituted, 5.39 *H*-1‴, 5.11 = *H*-1″), 4.14 – 3.59 (16 H + DS * 2 H, *H*-2, 3, 4, 5, 6 a, b + *H*-7 + *H*-2′,3′,4′,5′,6′a,b + *H*-2″,3″,4″,5″,6″a,b + *H*-2‴, 3‴, 4‴, 5‴, 6‴ a, b), 3.11 (3 H, *H*-9, *H*-1′), 1.96 (2 H, *H*-8).

2.3.3. Preparation for ESI-MS

2.3.3.1. Hydrolysis. Trifluoroacetic acid (900 μL, 2 M) was added to 1 mg of the modified glucan derivative. Hydrolysis was performed in a 1 mL V-vial for 2 h at 120 °C. The acid was removed under a stream of nitrogen and finally by co-distillation with toluene.

2.3.3.2. Methanolysis. Methanolic HCl (900 μL, 1.5 M) was added to 1 mg glucan derivative in a 1 mL V-vial. Methanolysis was performed for 2 h at 90 °C. After cooling to room temperature methanol was removed under a stream of nitrogen and the solid residue was several times re-dissolved with MeOH and evaporated to remove the acid.

2.4. Preparation of nano structures

2.4.1. Magnetic iron oxide nanoparticles

The iron nanoparticle dispersion (average diameter 12.2 nm ± 2.6 nm determined by image data processing of the corresponding TEM micrographs) was prepared as described (Fiege et al., 2012; Khalafalla & Reimers, 1980; Massart, 1981; Wotschadlo et al., 2009).

2.4.2. Polysaccharide nanoparticles

The polysaccharide derivative of interest was dissolved in 5 mL DMSO. Nanoparticles of modified glucans were prepared by dialysis against distilled water according to Hornig and Heinze (2008). Entrapping of iron oxide nanoparticles was achieved by adding various portions of this dispersion to the glucan solution prior to dialysis.

3. Results and discussion

3.1. Aminofunctionalization of cyanoethyl dextrans

As reported recently, we have prepared cyanoethyl dextrans (CED) and pullulans (CEP) in a wide DS range (Fiege et al., 2012). Derivatives with sufficiently high DS value (>2) formed nanoparticles by self-assembly during dialysis from of a DMSO solution against a poor solvent (water). To obtain amino-functionalized glucans for further coupling reactions, we reduced the cyano groups of various cyanoethyl dextrans with cobalt boride, prepared from cobalt chloride and sodium borohydride in aqueous solution (or after first dissolving the CED in 1 mL DMSO in case of water insoluble cyanoethyl dextrans) as has been reported (Gonera, 2004; Gonera et al., 2002; Verraest, da Silva et al., 1996; Verraest, Zitha-Bovens et al., 1998). Products were isolated and purified by dialysis after dissolving the residual cobalt compounds with diluted HCl. Completely or partial reduced aminopropyl glucans were obtained, characterized and nanostructuring of these derivatives was tested. ^{1}H NMR spectra of an aminopropyldextran (DS_{AP} = 0.64) and the antecedent cyanoethyldextran are shown in Fig. 1.

DS_{AP} estimation from ^{1}H NMR spectra was possible (Eq. (1)). The signal at 3.15 ppm is assigned to the methylene group adjacent to the amino function (CH_2—NH_2). The second methylene group is shifted from 2.82 ppm (CE) to 2.00 ppm (AP, CH_2—CH_2—NH_2). The protons of the remaining methylene group (linked to the sugar oxygen) are found in the range of the sugar ring protons at 3.35–4.25 ppm. The signal of the anomeric protons of aminopropyl dextran (APD) occurs at 4.97 and (in case of 2-*O*-substitution) at 5.21 ppm. From the NMR spectra recorded in aqueous solution, the average DS_{AP} for APD was calculated from the ratio of the integrals of the averaged two resolved methylene groups (8 and 9) of the aminopropyl residues to the summarized integrals of *H*-1 (Eq. (1)). For the remaining nitrile groups, Eq. (2), referring to the methylene groups adjacent to the nitrile group at (2.82 ppm), was applied. In case of the product with high DS_{CE} (APD-8) DMSO-d_6 had to be used as solvent in NMR measurements which caused less resolved and more complex spectra. Integration of the region of the anomeric was not possible due to the OH resonances overlapping with those of *H*-1. Alternatively, the DS_{AP} could be estimated from the integrals of the aminopropyl methylene groups in relation to the sugar ring-protons which were corrected for the contribution of the *O*-linked CH_2 groups of CN and AP, according to Eq. (3). The sum of the so estimated DS_{AP} and DS_{CE} of these partially reduced dextrans was higher than the total DS of the starting material. An overestimation of the protons of the more flexible substituents compared to the less mobile dextran backbone is assumed, as known from e.g. higher substituted fatty acid esters of starch. Since the integrals of the methylene protons are subtracted from the sugar ring protons for correction, these become additional underestimated. Since this underestimation of the glucose protons refers to both, CE and AP in the same way, the DS values were normalized to the starting DS. This does not affect the degree of conversion D_{red}, which was 15–16% for APD-8. D_{red} could be easily calculated applying Eq. (4).

Since fully reduced CE glucans no longer formed nanostructures even at DS > 2, only partial conversion of cyanoethyl to aminopropyl (AP) was also performed for these CEDs (see Table 1). The degree of reduction (D_{red}) ranged from 15% to 100%.

3.2. Functionalization of APD with aldehydes by reductive amination

Reductive amination of aldehydes with APD as the amino component was applied to link short sugar side-chains or bioactive molecules to the polymeric backbone. Thus, new properties like UV activity, fluorescence, antioxidant activity or molecular recognition sites can be introduced. Packaging foils from such type of materials could be used for contact preservation of food or cosmetics preventing release of active compounds in the environment. Addition to polymers can be applied to protect the polymer against radical-induced aging and loss of properties, while in packing applications it might act as protective for the content at the surface, e.g. of food. In both cases, release of active compounds or migration is unwanted. Conjugation by reductive amination is a less toxic alternative to click reaction to avoid toxic copper catalyst.

In the first, reversible step of a reductive amination the amino group reacts with the aldehyde followed by elimination of water to a Schiff base. In the subsequent and irreversible step, hydride is transferred to the iminium ion yielding a secondary amine. The reaction scheme and the employed aldehydes are shown in Fig. 2.

In our work vanillin was used because of its UV activity. *N*-Vanillyl chitosans and 4-hydroxybenzyl chitosans possess antifungal effects (Jagadish et al., 2012). As an representative of antioxidants 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde was coupled. This substance is used as additive in the synthesis of synthetic polymers. Antioxidative polymers have been used as polymer beads to prevent the oxidation of linoleic acid. An advantage is their easy separation from food oil after usage (Yamaguchi, Itoh, Ishikawa, & Kusuda, 1993).

Coupling short sugar chains like maltose by reductive amination leads to pseudo branching. Length and density of sugar arms (brushes) e.g. of linear glycans as pullulan can be modified and

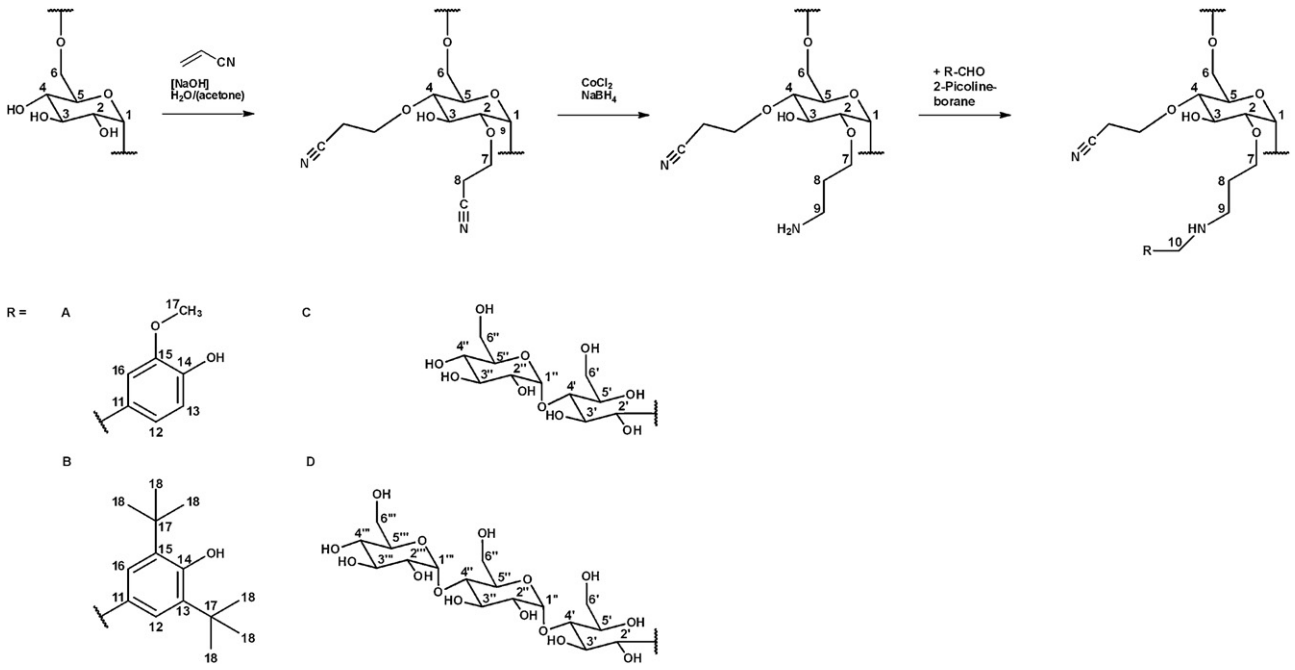

Fig. 2. Reductive amination of various aldehydes with *O*-aminopropyl-*O*-cyanoethyl dextran in methanol; (A) 4-hydroxy-3-methoxy-benzaldehyde, (B) 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde, (C) maltose, (D) maltotriose; reducing agent: picoline borane. Reaction is examplified for partial reduction of a 2,4-di-*O*-cyanoethyl glucosyl unit of CED. For degree of amino group conversion in reductive amination see Table 2.

thereby the solution properties of the polymers. To demonstrate the possibility of artificial branching of glucans with short sugar residues we used maltose and maltotriose.

Coupling of various aldehydes to the amino functions of the modified dextran was performed as summarized in Table 2. The APD were dissolved (or dispersed in case of high substituted cyanoethyl dextrans) in methanol and subsequently aldehyde was added. The pH was adjusted with HCl or NaOH (0.1–1 M, see Table 2). The reaction mixture was stirred at 45 °C for various periods. 2-Picoline borane complex was used in excess in two or more steps (Jagadish et al., 2012; Sato et al., 1998; Unterieser & Mischnick, 2011). The product was isolated by dialysis against water. Covalent coupling was confirmed by NMR and ESI-MS experiments. Degree of conversion of NH_2 to NHR and NR_2 groups was determined from ^{1}H NMR spectra: The protons (x) of the introduced aromatic residues were related to the protons of the methylene group according to Eqs. (5) and (6).

In case of maltose or maltotriose the new *H*-1 signal of the α-1,4-linked glucose residue of maltitol (5.11 ppm) was divided by the integral of the methylene group (2.00 ppm) (Hoffman & Davies, 1988). Coupling of the oligosaccharides to the amino dextran is proved by signal C1′ at 53.47 ppm.

The HMBC spectrum in Fig. 3 illustrates the success of reductive amination for vanillin (Table 2, Van-13). The former aldehyde carbon atom has been transformed into the covalently linked methylene group (*H*-10). The cross signal with *C*-12 is found at 4.08 respective 124.40 ppm.

The covalent linkage of various aldehyde residues bound to the amino groups of modified dextrans could also be confirmed by ESI-MS. After depolymerization of the glucan, *N*-mono- and *N*-disubstituted *O*-aminopropyl glucoses were detected as $[M+H]^+$ beside glucoses with additional unsubstituted aminopropyl groups (see Fig. 4a and b). In case of the coupling product with vanillin, trifluoroacetates as side products from hydrolysis with TFA occurred (* in Fig. 4a). The main methanolysis products of APD coupled with maltose, *N*-(1-deoxy-D-glucitol)-substituted methyl *O*-(3-aminopropyl)-glucosides, were detected as $[M+H]^+$ at m/z 416. Thus the successful reductive amination of APD with maltose (Fig. 4c) was also confirmed. Methyl *O*-aminopropyl-glucosides without maltose coupling could be detected at m/z 252.

Usually the aldehyde is the substrate (e.g. the reducing end of mono- or oligosaccharides) in reductive amination reactions of carbohydrates. Excess of primary amino reagent is usually applied to prevent formation of tertiary amines and cross-linking. In our case, the amino groups along the polymer chain are the substrates which shall be coupled to aldehydes with high yield. Thus, no excess of the amino component can be applied.

Sun et al. described kinetic and thermodynamic theories for the reaction of primary amines with the reducing end of polysaccharides (Sun, Wei, & Wei, 2009). According to them temperature and pH adjustment in relation to the pK_a value of the amino group are the most important parameters for a successful reaction. Sato et al. investigated reductive aminations of various aldehydes and ketones with a number of amines and picoline borane. They

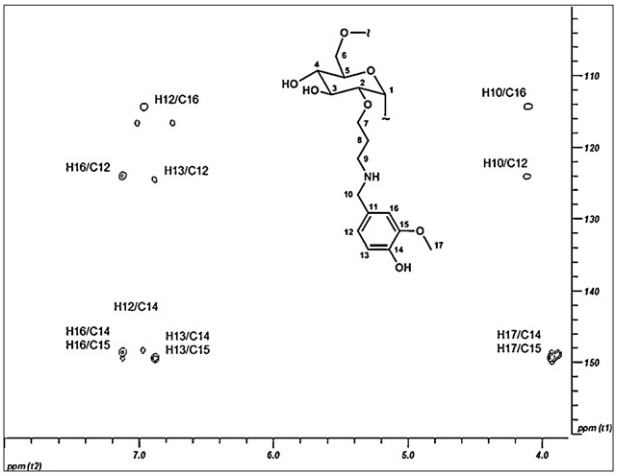

Fig. 3. HMBC (heteronuclear multiple bond correlation) spectrum of Van-13 (DS_{Van} 0.48, former DS_{AP} = 0.47) showing long range coupling. Cross peaks of C and H are assigned for some nuclei.

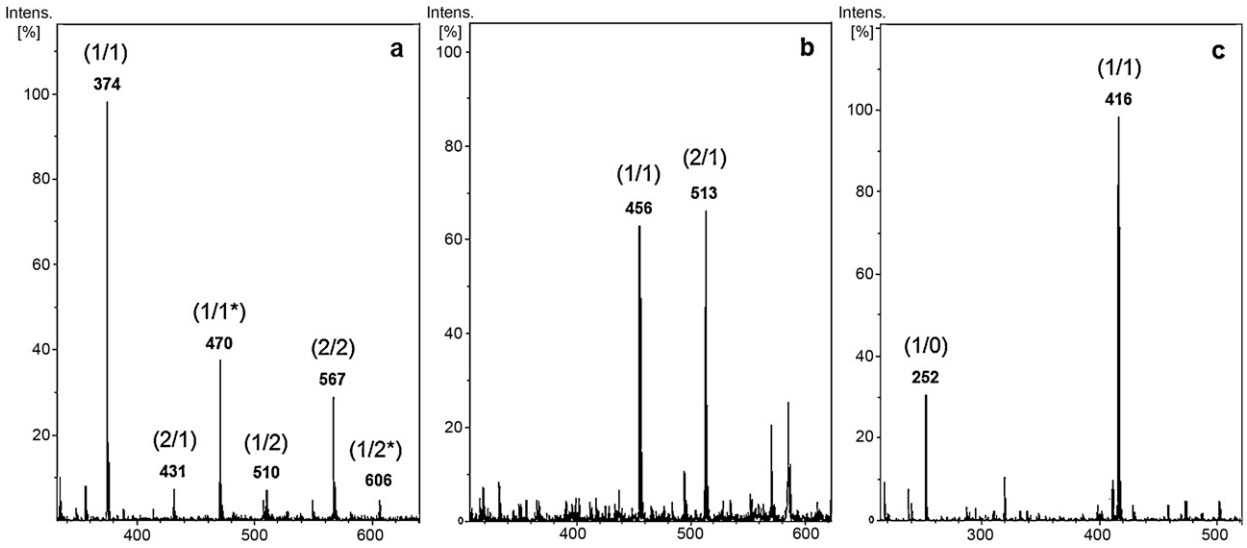

Fig. 4. ESI-MS spectra of *O*-aminopropyl glucose derivatives coupled with (a) vanillin (Table 2, Van-13), (b) BHT-CHO (Table 2, BHT-5), both after hydrolysis of the corresponding dextran derivatives with TFA, and (c) of methyl *O*-aminopropyl-glucosides coupled with maltose, obtained by methanolysis of dextran derivative G_2-8 (Table 2); in brackets: number of AP/number of coupled molecules; * for TFA side products.

used equimolar ratios of amine, carbonyl compound, and reducing agent and showed that reductive amination can be performed with high yields even without excess of amine and in aqueous media. The influence of water is not fully understood. Support of the hydrophobic association of amines and carbonyl compounds with the reductive reagent supporting the imine formation was suggested (Sato, Sakamoto, Miyazawa, & Kikugawa, 2004). Unterieser described the removal of solvent (water) in the work-up step as essential for a complete reaction (Unterieser & Mischnick, 2011). The labeling of oligosaccharides was investigated with picoline borane, which is a relatively cheap, mild, chemo-selective (no carbonyl reduction), stable and non-toxic reagent (Ruhaak, Steenvoorden, Koeleman, Deelder, & Wuhrer, 2010; Unterieser & Mischnick, 2011).

Our experiments show that the pH value has to be optimized for the respective aldehyde to get good conversion rates (see Table 2). While a reaction with vanillin at pH 4 led to 33–100% aldehyde coupling, nearly no reaction occurred at pH 8 (Table 2, Van-7). Stoichiometric amounts of vanillin could be bound in sample Van-13 at pH 4, and a DS_{Van} of 0.48 could be obtained (from DS_{AP} = 0.47). In contrast, sugar aldehyde hemiacetals required slight alkaline reaction conditions (pH 9), probably because the concentration of the free aldehyde form is thus enhanced, while the electrophilicity of the C=O is already enhanced by the many OH groups. The higher pH also shifted the equilibrium of the aminopropyl group toward the non-protonated side. However, it should be mentioned that reductive amination of carbohydrates with less basic aromatic amines is preferably performed at acidic pH. Appelhans et al. described alkaline conditions (0.1 M sodium borate) for the coupling of oligosaccharides with poly(ethylene imine) by reductive amination (Appelhans et al., 2009).

In sample BHT-5 (Table 2) an excess of the aldehyde (10 eq/NH_2) did not improve the degree of conversion of amino groups (12% conversion, DS_{BHT}: 0.15), while prolonged reaction times (53 h) were more effective (BHT-6) yielding a molar ratio of 0.53 for conjugated RCHO/amine (DS_{BHT} = 0.70). The latter reaction was performed using only 2 equivalents of BHT and the double molar amount of picoline borane.

Reductive amination with maltose or maltotriose required different reaction conditions. For entries G_2-1 – G_2-3 all with the same aminopropyl dextran (DS_{AP} = 0.46), different pH values were tested (3, 7 and 9). The best rate of aldehyde coupling was obtained at pH 9 (78%). Further experiments were carried out under these slightly alkaline conditions resulting in aldehyde coupling of 46–70% (see Table 2).

In summary, the reducing reagent should be added stepwise and the pH value of the reaction mixture has to be adjusted according to the aldehyde/amine pair. For the aromatic aldehydes used in this study pH 4 was favorable. For oligosaccharides in combination with the primary amino residues, the reaction medium should be adjusted to pH 9. The best way to increase the aldehyde coupling rate by reductive amination was prolonged reaction time.

3.3. Nanostructure preparation

As reported previously, highly cyanoethylated glucans (DS > 2) form nanospheres that can entrap ferromagnetic nanoparticles (Fiege et al., 2012). Based on these results we analyzed the self-assembly of cyanoethyl/aminopropyl dextrans with high DS_{CE} and a low degree of reduction (Table 1, sample APD-8, DS_{CE} = 2.31–2.35, DS_{AP} = 0.43–0.44). Products of the reductive amination with vanillin and BHT were submitted to the same procedure. All experiments were carried out with and without addition of ferromagnetic nanoparticles (abbreviation -Fe). The inorganic, magnetic cores (12.2 nm ± 2.6 nm determined by TEM measurements) were prepared by a precipitation process of Fe(II) and Fe(III) chlorides (molar ratio 1.7: 1.0) with aqueous ammonia solution as described (Fiege et al., 2012; Wotschadlo et al., 2009). Completely reduced cyanoethyl glucans are water-soluble, and no nanostructuring by self-assembly was observed when a solution in DMSO was submitted to dialysis against water. Also in the presence of iron oxide nanoparticles, no monodisperse nanostructures were detectable by dynamic light scattering. Instead, precipitation occurred, possibly caused by the metal complexing capacities of the amino groups that result in “crosslinking” (Daly & Munir, 1984).

Furthermore, APD reacted with vanillin or 3,5-di-*tert*-butyl-4-hydroxy-benzaldehyde were tested. All these glucan derivatives formed particles in the nano scale (see Table 3).

The particle size was also calculated by image data processing of the corresponding TEM micrograph. Fig. 5 shows TEM micrographs of sample NP-APD-8-Fe (a), NP-Van-16-Fe (b) and NP-BHT-6-Fe (c), all prepared in the presence of magnetic iron oxide nanoparticles.

Table 3
Parameters for nanostructure formation, results of DLS measurements.

Sample	Educt	DS_{CE}[a]	DS_{AP}[b]	DS_{RCHO}[c]	Iron NP[d] [μL]	Size/DLS	
						[nm][e]	[%][f]
NP-APD-8	APD-8	2.35	0.43	–	0	242	100
NP-APD-8-Fe	APD-8	2.35	0.43	–	20	203	100
NP-Van-14	Van-14	0.85	0.63	0.48	0	368	83
						78	17
NP-Van-14-Fe	Van-14	0.85	0.63	0.48	20	125	100
NP-Van-15	Van-15	0.91	0.49	0.45	0	489	82
						132	18
NP-Van-15-Fe	Van-15	0.91	0.49	0.45	20	157	98
						517	2
NP-Van-16	Van-16	2.31	0.44	0.19	0	237	100
NP-Van-16-Fe	Van-16	2.31	0.44	0.19	20	199	100
NP-BHT-5	BHT-5	–	1.31	0.15	0	307	97
						17	2
						5394	1
NP-BHT-5-Fe	BHT-5	–	1.31	0.15	20	192	99
						5266	1
NP-BHT-6	BHT-6	–	1.31	0.70	0	83	85
						17	15
NP-BHT-6-Fe	BHT-6	–	1.31	0.70	20	87	54
						25	38
						2201	8

[a] Remaining after reduction calculated from AP NMR.
[b] Calculated after reduction.
[c] DS value of coupled aldehyde, calculated by Eq. (6).
[d] 20 μL = 0.0133 mmol Fe.
[e] Diameter calculated from intensity distribution.
[f] % of intensity distribution.

In case of NP-APD-8-Fe the mean diameter was determined as 156 nm ± 45 nm by image data processing of the corresponding TEM image. Clustering of particles (see Fig. 5a and b) may be either caused by increasing concentration of particles from wet patches on the hydrophobic carbon foil because of insufficient surface wetting or by magnetic attractive clumping of the particles themselves or a combination of both effects. DLS measurements showed no agglomeration. The particle size for sample Np-Van-16-Fe was determined with 128 nm ± 35 nm (from TEM image). The DLS experiments of NP-APD-8 and NP-Van-16 with and without iron as well as NP-Van-14 with iron showed only one size fraction of coated nanoparticles. In the last sample an entire DS value of ≈1.5 (DS_{CE} + DS_{AP}) with approximately one third vanillin coupling is sufficient to form monodisperse particles. These glucans are supposed to have the appropriate hydrophobic–hydrophilic balance to form nanoparticles by self-assembling during dialysis. The TEM micrographs of sample NP-BHT-6-Fe (DS_{AP} 1.31 and 87% *N*-3,5-di-*tert*-butyl-4-hydroxybenzyl substitution) also showed nanostructuring (75 nm ± 14 nm by image data processing of the corresponding TEM image), but not the entire iron oxide nanoparticles were entrapped. The polysaccharide structures contain only low amounts of iron. Considering the requirements of amphiphilicity for nanostructuring, it can be assumed that in the spheric particles observed the hydrophobic regions of the modified polymer are orientated inwards and hydrophilic regions are directed to the outer water phase. An adverse aspect of the performed experiments is the occurrence of several nanoparticle size distributions in one experiment, e.g. sample NP-Van-15 (Table 3) resulting in a high polydispersity in some cases. Using simple size separation techniques like filtration, asymmetric flow field flow fractionation or centrifugation it should be possible to reduce the size heterogeneity. By addition of iron oxide nanoparticles, particle size and polydispersity decreased relative to those which have been prepared in the absence of ferromagnetic particles (exception:

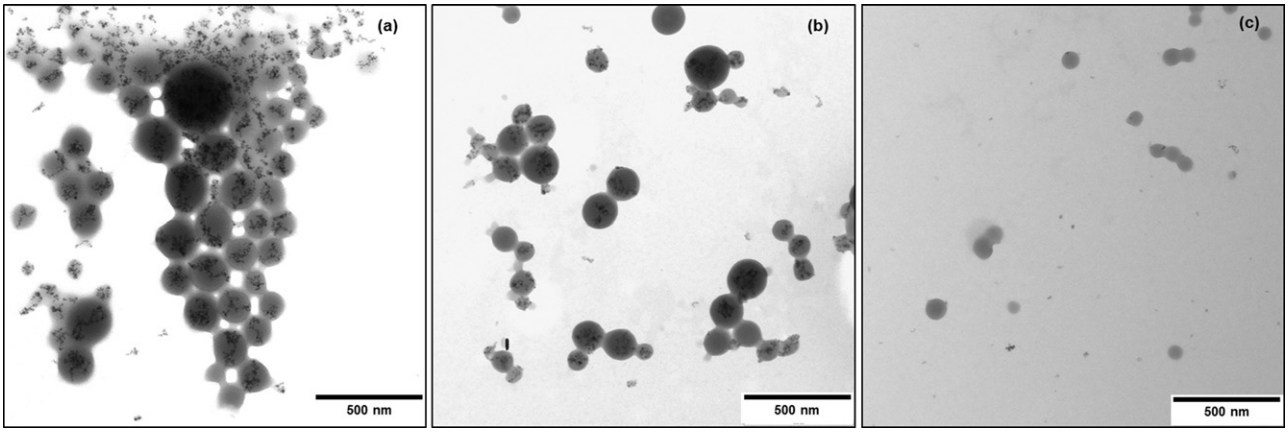

Fig. 5. TEM micrographs of nanostructured polysaccharides with iron oxide cores (20 μL iron oxide, respective 0.0133 mmol iron) of (a) aminopropyl cyanoethyl dextran (Table 3 sample NP-APD-8-Fe), (b) vanillin coupled to aminopropyl dextran (Table 3 sample NP-Van-16-Fe) and (c) BHT coupled to aminopropyl dextran (Table 3 sample NP-BHT-6-Fe).

NP-BHT 6). Both, hydrophobic properties and interaction of cyanoethyl groups with the iron oxide nanoparticles, explain the formation of polysaccharide coated metal oxide nanostructures (Fiege et al., 2012). The resulting multicore nanoparticles are of interest due to their UV activity, their antioxidant activity and their controllability by magnetic fields. Magnetic separation techniques or magnetic particle imaging may be potential applications. The quasi-branched dextrans (maltose or maltotriose side-chains) may be used in glucan arrays for the investigations of protein-carbohydrate interactions (Höbel et al., 2011; Lindhorst, 2011).

4. Conclusions

A suitable method for amino functionalization of glucans within wide DS range of the cyanoethyl precursors was presented. While pure aminopropyl glucans did no longer form nanostructures, only partial reduction could safe this property of high substituted cyanoethyl glucans. Coupling of various aldehydes including oligosaccharides (vanillin, BHT-CHO, maltose, maltotriose) by reductive amination was successful up to an average molar ratio of 1.03 residues/amino group as was proved by 2D NMR spectroscopy and ESI-MS. The pH of the reaction mixture was adjusted according to the chemistry of the aldehydes. The reductive amination with maltose or maltotriose required slight alkaline conditions (pH 9), while in case of aromatic aldehydes the coupling yield was increased at pH 4. Prolonged reaction time was superior compared to excess of reagents. Nanostructuring of these dextran derivatives by self-assembly with and without addition of iron oxide nanoparticle dispersion was investigated. Depending on the chemistry of the respective glucan derivative, a mono- or bimodal distribution of functionalized particles was obtained. At the appropriate substitution pattern, the ferromagnetic particles were entirely entrapped.

Formula

$$DS_{AP} = \frac{1/4(\int CH_2CH_2NH_2 + \int CH_2NH_2)}{\int H1 + \int H1_{2\text{-}O\text{-}CE} + \int H1_{2\text{-}O\text{-}AP}} \quad (1)$$

$$DS_{CE} = \frac{1/2 \int CH_2CN}{\int H1 + \int H1_{2\text{-}O\text{-}CE} + \int H1_{2\text{-}O\text{-}AP}} \quad (2)$$

$$DS_{AP} = \frac{1/4(\int CH_2CH_2NH_2 + \int CH_2NH_2)}{1/6(\int \Sigma(H2,3,4,5,6_{a,b}OCH_2) - 1/2(\int CH_2CH_2NH_2 + \int CH_2NH_2) - \int CH_2CN)} \quad (3)$$

$$D_{red}\,[\%] = \frac{DS_{AP}}{DS_{AP} + DS_{CE}} \quad (4)$$

$$\text{Degree of conversion } [\%] = \frac{(1/x \int H_{arom})}{1/2 \int CH_2} \cdot 100 \quad (5)$$

$$DS_{RCHO} = \frac{DS_{AP}}{100} \cdot \text{degree of conversion}\,[\%] \quad (6)$$

Acknowledgements

The authors thank Jun.-Prof. Dr. Georg Garnweitner, Institute for Particle Technology, TU Braunschweig, for the opportunity of using the DLS instrument, and Prof. Dr. Thomas Heinze, Friedrich-Schiller-Universität Jena, for the introduction in the preparation of ferrofluids. The authors acknowledge also Dennis Rosebrock (Institute for Robotics and Process Control, TU Braunschweig) for the image data processing program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

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