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Starch microspheres as carriers for X-ray imaging contrast agents: Synthesis and stability of new amino-acid linker derivatives

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Abstract

The relative stability of particulate contrast agents for X-ray imaging, consisting of a succinic-acid derivative of a water-soluble X-ray contrast agent bound to starch particles through an amino-acid ester bond, has been studied. To investigate the effect of chain length of the amino-acid linker on degradation rate, two glycine-linked derivatives and a corresponding β-alanine-linked derivative were prepared as model compounds. The cleavage rate of the amino-acid ester bond in the starch particle β-alanine derivative had a significantly lower cleavage rate than in the corresponding glycine derivatives; after 22 h in human blood serum at 37 °C the remaining fraction of the undegraded β-alanine-linked derivative was 78%, while 31.1 and 29.3% were the remaining fractions of the two glycine-linked derivatives. The cleavage data correlated well with biphasic cleavage processes with two distinct half lives for the respective pseudo first order processes. The second preparation with the glycine linker had a cleavage profile and rate equivalent to that of the first one in human blood serum, but the corresponding hydrolysis in phosphate buffer was significantly slower with 79.9%-remaining fraction after 22 h, and was apparently a monophasic pseudo first order reaction. Variation of the degradability of the starch matrix had apparently no significant effect on the cleavage rates of the linker. This suggests that components in the human blood serum catalyze the cleavage of the ester bond in these derivatives and that a fraction of the covalently bound contrast agent had a significantly slower cleavage rate from the matrix. The derivative with a glycine linker between the carrier matrix and the contrast agent is a promising candidate for liver and spleen directed X-ray contrast with respect to density of contrast generating iodine and biodegradability.

Keywords: X-ray imaging; Contrast agents; Polysaccharides; Amino-acid linker

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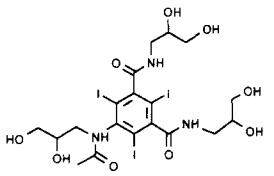
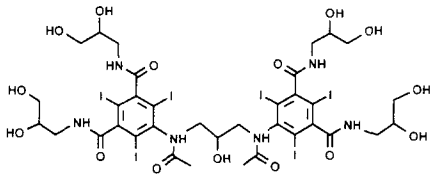
1. Introduction

X-ray contrast agents in routine clinical use, such as iohexol (**1**) and iodixanol (**2**) [1,2], are water-soluble hydrophilic compounds with low osmolality. These agents have an extracellular and extravascular distribution and renal elimination. They are often referred to as general contrast agents due to the unspecific biodistribution and their broad application area.



(1) - iohexol



(2) - iodixanol

Although the main indications for the general contrast agents is diagnosis of diseases related to the cardiovascular and renal systems, these agents are also used for enhancing detection of pathology in computed tomography (CT) of the liver. However, they are not ideal for such indications since they diffuse and partly accumulate in both normal and pathological tissue [3], and are rapidly cleared from the blood and tissue [4]. Opposite to these water-soluble contrast agents, particulate contrast agents are taken up intracellularly by normal liver tissue but not by pathological tissue [5]. As early as 1923, particulate contrast agents for X-ray imaging were used in cholecystography, when tetrabromophenolphthalein particles were administered intravenously [6]. Since that time, many concepts for contrast enhancement of the reticuloendothelial system have been evaluated, including emulsions [7], organic particles [8], and liposomes [9]. Biodegradability of the matrix and excretion rate of the contrast agent moiety are important parameters for the clinical safety of such products. Microparticles prepared by precipitation of a water-insoluble derivative of metrizoate, proved to be rapidly excreted from the liver [10]. Contrast agents covalently attached to particles have been suggested as potential contrast agents for the reticuloendothelial system, i.e. in liver and spleen [5].

The starch particles used in this study can be prepared within well-defined limits of particle characteristics, degree of cross-linking, and biodegradability [11]. Few reports describe the use of similar particles as carriers of contrast agents for diagnostic imaging. We have previously described water-soluble polysaccharides and cross-linked starch microspheres as carriers of paramagnetic contrast agents for magnetic resonance imaging (MRI) [12–14]. As a continuation, the aim of this study was to synthesize and evaluate the relative in vitro stability of some ester derivatives of particulate polysaccharide carriers of X-ray contrast agents, and identify linkers where the stability is suitable to provide clinical useful X-ray contrast enhancement in the reticuloendothelial system and quick elimination after imaging.

2. Experimental

General.—Fluorenyl-methyloxycarbonyl glycine and fluorenyl-methyloxycarbonyl β-alanine (FMOC-glycine and FMOC-β-alanine) were purchased from Novabiochem AG. *N*-(3-Dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride (EDC) and all other chemicals were purchased from Fluka Chemie AG or Merck. Human blood serum was purchased from The Norwegian Red Cross Center, Oslo. DMF and other organic solvents were dried by storage over 4 Å molecular sieves. Water was distilled before use.

Spherical starch particles with various average diameters were prepared by cross-linking hydrolysed potato starch with epichlorohydrin by a modification of the bead polymerization method [15]. The particles were estimated to have a mean number of glucose unit equivalents of 5.5 mmol/g of dry solid. The size of the particles was measured with a Coulter Counter.

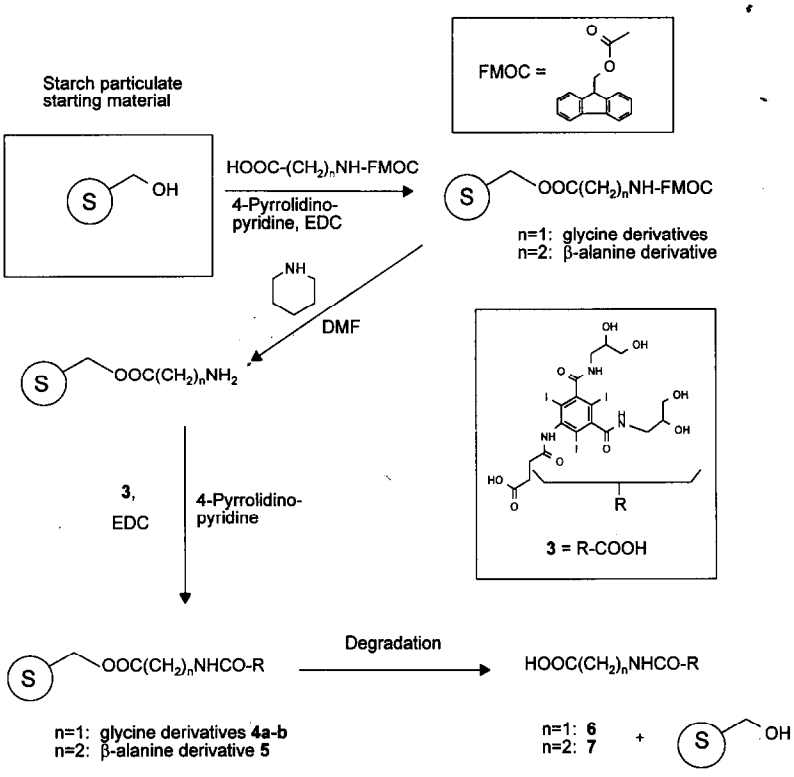
Ultrasound treatments were carried out by sonication in an ultrasonic bath at 20 kHz and 50 W. Analysis of water content was performed using the Karl Fischer titration method. HPLC analysis was performed using a Hewlett–Packard or a Perkin–Elmer System, an LC-15B absorbance detector and a RP-18 column. Eluents are given where necessary.

The starting material 5-amino-3,5-bis-(2,3-dihydroxypropylcarbamoyl)-2,4,6-triiodo-benzene for the preparation of **3** is an intermediate from the synthesis of **1**, and was required from Nycomed Imaging A/S.

HPLC analysis of the batch sample used in this work was performed using a gradient of aq 1–13% CH_3CN, and indicated > 99% purity. Iodine anal. (fluorescence spectrophotometry), found: 54.1%. Calcd: 54.0%.

N-[3,5-bis-(2,3-Dihydroxypropylcarbamoyl)-2,4,6-triiodo-phenyl]-succinamic acid (**3**).—5-Amino-3,5-bis-(2,3-dihydroxypropylcarbamoyl)-2,4,6-triiodo-benzene (200 g, 840 mmol) and 4-*N,N*-dimethylamino-pyridine (1.80 g, 14.4 mmol) were suspended in acetic anhydride (1 L) at ambient temperature. The suspension was stirred for 2 h at 70 °C, followed by 3 h at 80 °C, and then at ambient temperature for 15 h. The clear yellow solution of tetra-*O*-acetyl-5-amino-3,5-bis-(2,3-dihydroxypropylcarbamoyl)-2,4,6-triiodo-benzene was concentrated to dryness at 60 °C under reduced pressure, and the residue was dried in vacuo at 50 °C. The solid was washed as a slurry with ether (700 mL) in portions, and dried in vacuo overnight at 50 °C to yield 240 g yellowish material. A part of the solid (100 g) was dissolved in dimethylacetamide (200 mL), the solution was cooled to 2 °C in an ice/water bath and succinic-acid chloride monomethyl ester (28.0 g, 229.0 mmol) was added dropwise during 35 min. After 50 h at ambient temperature the solvent was removed at reduced pressure, the residue was dissolved in MeOH (640 mL), and 5 M NaOH (194 mL) and water (100 mL) were added. The solution was stirred for 5 h at 50 °C at pH 12, and kept overnight at room temperature, then neutralized with 5 M HCl. Rotary evaporation yielded a yellowish residue which was dissolved in water (600 mL). After concentration, the solid was dissolved in water (240 mL), the solution was filtered, and the pH in the filtrate was adjusted from 6.2 to 1.6 using 12 M HCl. A slow cooling to 18 °C initiated precipitation. Stirring for 48 h, filtration, washing on a filter with cold 0.1 M HCl (4 × 15 mL) and drying in vacuo at 50 °C yielded 62.9 g (68%) white solid; mp above 300 °C (decomp.); ^{13}C NMR (75.5 MHz, Me_2SO-d_6; Me_4Si, δ 0): δ 173.32 (*C*OOH), 169.47 (2 aryl–*C*O), 169.24 (aryl–NH*C*O), 149.92 (aromatic *C*-carbonyl), 149.88 (aromatic *C*-carbonyl), 142.92 (aromatic *C*-amidoyl), 99.11 (*C*–I *ortho* to aromatic C-amidoyl), 98.93 (*C*–I *ortho* to aromatic C-amidoyl), 90.07 (*C*–I *para* to aromatic C-amidoyl), 69.84 (2 *C*H–OH), 63.84 (2 CH_2–OH), 42.56 (2 CH_2–NH), 30.61 (CH_2–carboxyl), and 28.95 (CH_2–carbamoyl). Anal. Calcd for $C_{18}H_{22}I_3N_3O_9$: C, 26.85; H, 2.75; I, 47.29; N, 5.22. Found: C, 26.56; H, 2.89; I, 47.08; N, 5.36.

{3-[3,5-bis-(2,3-Dihydroxypropylcarbamoyl)-2,4,6-triiodo - phenylcarbamoyl] - propionylamino} - acetic acid starch ester particles, glycine derivatives **4a** *and*



Scheme 1. Synthesis, structure and cleavage of derivatives **3**, **4a**, **4b**, and **5**.

4b, *and 3-{3-[3,5-bis-(2,3-dihydroxypropylcarbamoyl)-2,4,6-triiodo-phenylcarbamoyl]-propionylamino}-propionic acid starch ester particles, β-alanine derivative* **5**.—The following general procedure was applied in the preparation of the products **4a**, **4b**, and **5** in 60 mmol scale (Scheme 1): Cross-linked starch particles were swollen for several hours in DMF (20 mg particles/mL) and then washed repeatedly with DMF. After resuspension in DMF (20 mg/mL) the suspension was treated ultrasonically for 15 min. The following reagents in equivalents per glucose units in starch particles were successively added: FMOC-amino acid (1.0 equiv), EDC (1.0 equiv), and 4-pyrrolidinopyridine (0.1 equiv). The suspension was treated ultrasonically for 5 min. After 4 h shaking the suspension was washed 5 times with DMF (50 mL/g particles) by successive centrifugation/resuspension. The product was resuspended in DMF (50 mL/g particles), and piperidine (20% of DMF volume) was added. After stirring for 50 min and shaking for 50 min at ambient temperature, the suspension was washed as described above with the use of 20 kHz ultrasound to speed up the mixing.

N-[3,5-bis-(2,3-Dihydroxypropylcarbamoyl)-2,4,6-triiodo-phenyl]-succinamic acid (**3**) (1:1 equivalents to the number of glucose units in the particles) was dissolved in DMF (1:4 w/w) at 60 °C. The resulting cooled solution was added to the particle suspension at ambient temperature, followed by the addition of EDC (1.1 equiv) and 4-pyrrolidinopyridine (0.1 equiv). The suspension was treated ultrasonically for 15 min and shaken for 18 h at ambient temperature. After centrifugation, resuspension and repeated centrifugation, the residue was dried in vacuo at 50 °C. For starting material properties and analysis of **4a**, **4b**, and **5**, see Table 1.

Test for reactivity of **3** *with starch particle hydroxyl groups*.—The succinic-acid derivative **3** was reacted with starch particulate starting material without amino acid groups on the matrix using all relative amounts of particles, **3**, EDC, and 4-pyrrolidinopyridine, whereby concentrations and conditions were as in the general procedure described above. Result: analysis of iodine in the product, < 0.4% I.

Table 1
Analysis of **4a**, **4b**, and **5**

Sample	Properties of starting materials		Analysis of iodine in products (%)
	Mean diameter (μm)	$t_{1/2}$ (α-Amylase [a])	
4a	1.4	25 min	20.7
4b	1.0	2–3 min	24.9
5	1.4	25 min	22.2

[a] α-Amylase degradation of unreacted starch particles (starting material) – $t_{1/2}$ at 4.0 μkat/L [11].

{3-[3,5-bis-(2,3-Dihydroxypropylcarbamoyl)-2,4,6-triiodo-phenylcarbamoyl]-propionylamino}-acetic acid **6**.—*N*-[3,5-bis-(2,3-Dihydroxypropylcarbamoyl)-2,4,6-triiodo-phenyl]-succinamic acid (**3**) (8.28 g, 10.3 mmol) was dissolved in DMF (60 mL) at 50 °C. After filtration, glycine *t*-butyl ester (1.48 g, 11.3 mmol) and EDC (2.17 g, 11.3 mmol) were added, and the mixture was stirred for 23 h at ambient temperature. The solution was concentrated to dryness at 60 °C under reduced pressure and the residue was dried in vacuo at 50 °C. A solution of the residue (8.2 g) in water (25 mL) at pH 6, was loaded on a preparative RP-18 column, and the column was eluted with aq 30% MeOH yielding **6** (3.97 g, 42%) as a solid, purity > 97.8% (HPLC); mp above 300 °C (decomp.). Anal. Calcd for $C_{20}H_{25}I_3N_4O_{10}$: C, 31.39; H, 3.62; I, 41.46; N, 6.10. Found: C, 31.59; H, 3.85; I, 40.40; N, 6.40. The sample was used without further purification as an external standard in the cleavage studies of the particle derivatives after correction for impurities. The expected product (**7**) from cleavage of the corresponding β-alanine derivative (**5**) was not prepared.

Methods for evaluation of stability.—The rate of cleavage of the particulate derivatives **4a**, **4b**, and **5** in human blood serum and phosphate buffer was calculated from the ratio between the amount of the respective anticipated cleavage products **6** or **7** in the particle suspension media, and the remaining amount of substituent attached to the carbohydrate carrier versus time. All analysis of kinetics of the cleavage reactions, rate constants and half lives were performed using the computer program Siphar® version 4.0. Curve fitting was performed using the computer program Sigmaplot® version 1.02.

The concentration of the cleavage products, the free amino acid derivatives **6** or **7** in human blood serum or phosphate buffer at 37 °C and physiological pH, was monitored using HPLC. The pH in the phosphate buffer used for the study of **4b** was 7.00, and in the human blood serum used for the study of **4a**, **4b**, and **5** it was 7.74. The concentration of particles in all studies was 5 mg/mL.

The aliquots from the stability studies of **4a**, **4b**, and **5** in human blood serum were centrifuged. The supernatants were mixed with aq 50% trichloroacetic acid (10%) to remove proteins and recentrifuged. The amino acid derivative **6** was used as an external

standard for **4a** and **4b** after correction for impurities. For the stability studies in human blood serum at 37 °C, 10 mM CF_3COOH in aq 7% MeOH was used as HPLC eluent.

Iohexol (**1**) was used as an internal standard in the cleavage studies of **4b** in phosphate buffer. To separate **1** from **6** in the HPLC analysis of the samples in these studies, they were eluted with an aq 1–4% CH_3CN gradient for 30 min, followed by an aq 4–20% CH_3CN gradient for 15 min. The pH in both eluents was kept in the range 2.0–2.4. External standards for calibration of the HPLC system were prepared by dissolving **6** in human blood serum or phosphate buffer. The concentration of **6** or **7** from the cleavage studies was calculated from the standard peak areas. Based on our earlier experience with analysis of various X-ray contrast agents, it was assumed that **7** had the same molar extinction coefficient as **6**. The difference in molecular mass between **6** and **7** was compensated for by multiplying the integrals with the ratio between the respective molecular masses.

Experimental errors are undoubtedly introduced from sources like impurity of the external standard (**6**), handling of the particle samples with respect to homogeneity or calibration errors in the analytical measurements. However, the aim with the present studies was to determine relative cleavage half lives and not to generate absolute kinetic information. Thus, half lives and rate constants are given as approximate values.

3. Results and discussion

The results of the stability studies are summarized in Table 2 and illustrated in Figs. 1 and 2.

The analysis of the data for the degradation of **4a**, **4b**, and **5** is given in Table 2. The HPLC peak for the cleavage product from **4a** and **4b** was concurrent with the peak for **6**. It was assumed that the peak observed in the chromatograms from degradation of **5** represented **7**. The data for degradation in human blood serum are consistent with biphasic processes with two pseudo first order rate constants for both **4a**, **4b**, and **5**. The β-alanine derivative (**5**) has a significantly lower cleavage rate than the corresponding glycine derivatives (**4a**, **4b**); after 22 h in human blood serum at 37 °C, the remaining fraction of **5** was 78% while 31.1 and 29.3% were remaining fractions of **4a** and **4b**, respectively (Figs. 1 and 2, Table 2).

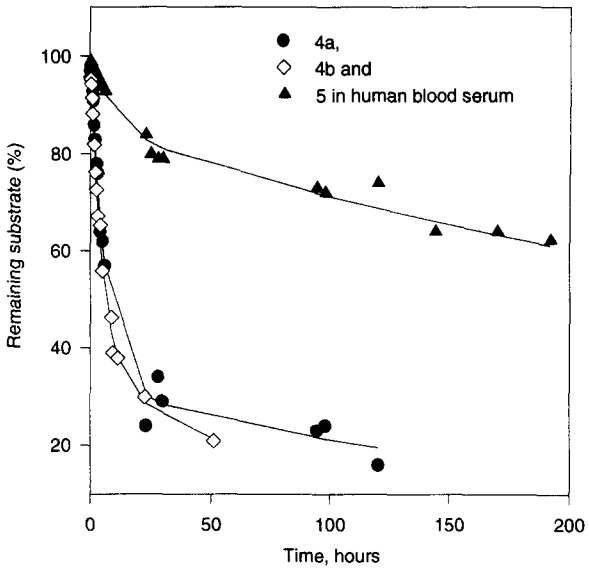


Fig. 1. The cleavage of **4a**, **4b**, and **5** in human blood serum at 37 °C.

Conformational differences as a result of a different chain length of glycine and β-alanine is anticipated to give minor contributions to the difference in half life. The major effect is expected to be the contribution from the electronic influence from the amino group in the amino-acid linkers. This is also

Table 2
Results of ester bond stability studies for derivatives **4a**, **4b**, and **5**

Sample	Suspension medium [a]	Approximate half lives and apparent 1st order rate constants				Remaining matrix-bound fraction after 22 h (%)	Corr.
		First phase		Second phase			
		$t_{1/2}$ [b] (h) (pts) [c]	k_1 [d] (h^{-1}) $\times 10^{-3}$	$t_{1/2}$ [b] (h) (pts) [c]	k_2 [d] (h^{-1}) $\times 10^{-3}$		
4a	A	4.3 (12)	161.2	169.3 (7)	4.1	31.1	0.9958
4b	B	112 (17)	6.2	–	–	79.9	0.9979
4b	A	2.9 (12)	238.9	46.8 (4)	14.8	29.3	0.9971
5	A	8.6 (12)	80.6	391.7 (11)	1.8	78.0	0.9943

[a] The particle concentration in suspension was 5.0 mg/mL in all cases. A: human blood serum; B: phosphate buffer.
[b] Approximate half life in cleavage of contrast agent from particle matrix.
[c] Number of measuring points representing the phase (see Fig. 1Fig. 2).
[d] Apparent 1st order rate constant.

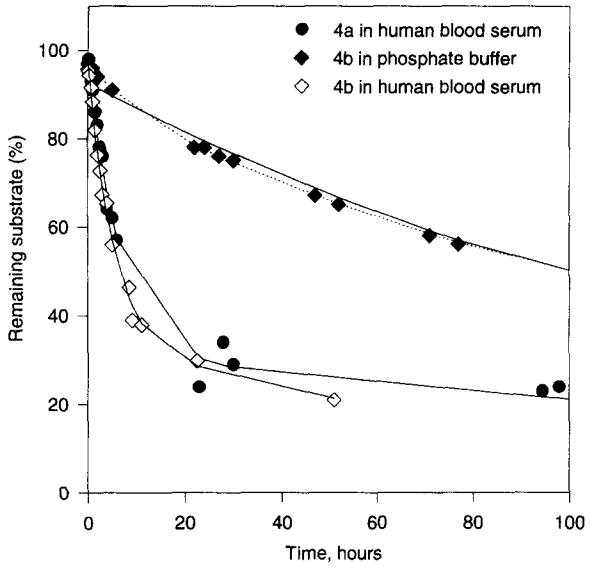


Fig. 2. The cleavage of **4a** and **4b** in human blood serum, and **4b** in phosphate buffer pH 7.00 at 37 °C.

reflected in the difference in carboxylic pK_a's for glycine and β-alanine (2.34 and 3.60, respectively [16]) and the fact that the methyl β-alaninate is significantly more hydrolytically stable than methyl glycinate [17].

It is a significantly higher rate constant for **4a** and **4b** than for **5** in both phases which is to be expected if the stability of the amino-acid ester bond lysis is the dominating process in the release of water-soluble contrast agent to the supernatant. The second phase half life for **4b** is significantly shorter than the second phase half life for **4a**. This is probably related to the lack of measuring points for **4b** between 60 and 100 h (Fig. 2); it is believed that the cleavage profile in the second phase for **4a** and **4b** would have been similar with these points included.

The curve fit for the cleavage data for **4a**, **4b**, and **5** in human blood serum follows a biexponential decay. This is a typical mathematical description of a compartmentalization, where a fraction of the covalently bound contrast agent experiences different conditions for the ester hydrolysis. The corresponding semi-logarithmic plots for **4a**, **4b**, and **5** are not linear. The hydrolysis of **4b** in phosphate buffer (Fig. 2) is monophasic, significantly slower and apparently follows pseudo first order kinetics. There is no significant difference between a monoexponential (dotted line) and a biexponential (solid line) fit for **4b** in phosphate buffer (Fig. 2); the corresponding semi-logarithmic plot for **4b** is linear. The difference in pH between the phosphate buffer and the human blood serum (7.00 and 7.74, respectively) may result in a higher lysis rate in the second medium. In a study [18] of ester-bond cleavage in a water-soluble dextran derivative where metronidazole was linked to dextran 70 000 via a succinic-acid linker, the cleavage rates in phosphate buffer only had a 2.5-fold ratio between at pH 7.0 and 7.4. The ratio between the cleavage rates of **4b** in phosphate buffer and human blood serum in the first phase is more than 38 (Table 2). This suggests that components in the human blood serum may catalyze the cleavage of a fraction of substituent in these derivatives. The normal concentration of α-amylase and calcium in the human blood serum is about 4–6 μkat/L and 2–3 mmol/L, respectively. These values are in the same range as the concentration of the calcium-activated enzyme used to estimate the degradation rate of the starch matrix [11]. However, there is no evidence that degradation of the starch particle matrix by α-amylase is affecting the linker cleavage significantly over the time scale in Fig. 2, as a consequence of better accessibility for water and enzymes [13]. Supporting this is the fact that the cleavage profile for **4a** and **4b** in human blood serum did not deviate significantly (Fig. 2), even if the starting material matrix in **4b** had a much shorter half life in the presence of α-amylase and calcium than **4a** (Table 2). The other common enzymes and proteins in human blood serum are also anticipated to influence the rate of cleavage of the ester bond in the linker. In the study of the water-soluble metronidazole dextran derivative [18] the cleavage rate in 80% human plasma and buffer at 37 °C was approximately the same for this dextran derivative, concluding that no catalysis of cleavage from components in plasma could be observed. However, it is a fundamental difference between the dextran derivatives described in the referred work and the heterogeneous system studied in the present work. Upon interpretation of the results of the cleavage studies in human blood serum one needs to take into account the nature of the starch particles used in this work. They are not rigid, solid particles in suspension, but swell and behave like gels in hydrophilic or water-containing media. Thus, solutes of more low-molecular nature may swell into the particles, and the chemical derivatization of the carbohydrate hydroxyl groups described above will also occur internally in the particles. It is questionable whether diffusion of water inside the particles is significantly slower than outside. However, diffusion of macromolecular solutes such as proteins in human blood serum into the particles is expected to be low. This may lead to lower rates of hydrolysis, catalyzed i.e.

by serum components inside the particle structure than observed for an ester derivative dissolved in the serum. Consequently, this may explain the biexponential behavior of the cleavage profile in human blood serum. Another possibility is different hydrolysis rates as a result of substitution at both primary and secondary carbohydrate hydroxyl groups possibly in combination with sterical hindrance for nucleophiles or catalysts. The starch matrix has a primary and two secondary hydroxyl groups at C-6, C-2, and C-3 as possible substitution sites, and the decreasing rate of hydrolysis of esters with increasing branching at the alcohol part is known from chemistry text books [19]. However, more studies related to the structure of these derivatives are needed to investigate these questions properly, and that is beyond the scope of the present study.

In conclusion, suitable linker units for spacing the contrast agent away from the ester bond, a method for their preparative use, and derivatives (**4a, 4b**) with a reasonable overall half-life in human blood serum have been identified. The derivatives with a glycine-linker are promising candidates for liver and spleen directed X-ray contrast with respect to content of contrast agent and biodegradability. Degradation may yield the biodegradable starch matrix, amino acid derivatives, and the chemically inert X-ray contrast agent derivative.

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