Spin Labeled Bovine Serum Albumin, Spin Labeled Bovine Serum Albumin Chelating Agents and Their Gadolinium Complexes. Potential Contrast Enhancing Agents for Magnetic Resonance Imaging

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Bovine Serum Albumin, Gadolinium Complexes, Contrast Agents for NMR Imaging, Nitroxyls

Bovine Serum Albumin (BSA) was spin labeled using the active esters **3** and **5** containing a nitroxyl radical to give the spin labeled BSA derivatives **4** and **6.** Spin labeled CDTA, DTPA and TTHA chelating agents **10, 13** and **16** and the corresponding BSA derivatives **17, 18** and **19** and their gadolinium complexes **20, 21** and **22** were synthesized. All these BSA derivatives were evaluated as possible contrast enhancing agents for NMR imaging by measurement of spin-lattice (T_1) and spin-spin (T_2) relaxations and by calculations of relaxivities *i.e.*, slopes of $1/T_1$ *versus* concentration plots. The spin labeled BSA-complexon-gadolinium conjugates **20, 21** and **22** were found to have better relaxation times and relaxivities than the BSA spin labeled derivatives **4, 6, 17, 18** and **19.** The spin count for the BSA derivatives **4, 6, 17, 18** and **19** and for their gadolinium conjugates **20, 21** and **22** was found to be between 9–10 spins/molecule of the protein. The gadolinium conjugates **20, 21** and **22** of spin labeled BSA possess high relaxivities combined with a nitroxyl marker that could be used with EPR to assess pharmacokinetic behaviors of these potential contrast agents.

Introduction

Paramagnetic compounds, including nitroxyl radicals and metal ion chelates, have been recently evaluated as pharmaceuticals to enhance contrast on NMR images [1–5]. For the most part, compounds have been selected for intravenous administration that distribute non-specifically in the vascular and extracellular fluid spaces and are renally excreted. This non-specific pattern of distribution and excretion has been found useful for contrast enhancement on NMR images of kidneys, inflammatory lesions, and breakdown of the blood-brain barrier [1, 2, 3, 6, 7]. The development of one such metallo-complex, gadolinium-DTPA, has advanced to the stage of clinical trials [6, 7].

To achieve more specific targeting of paramagnetic contrast agents, attempts have been made to conjugate metallo-chelates to biomolecules including serum albumin and monoclonal antibodies [8–11]. Unique features of serum albumins include (**1**) a biodistribution primarily in the vascular space following intravenous administration and (**2**) a remarkable ability to bind reversibly a wide variety of organic compounds [12, 13]. The predominant intravascular distribution of large molecular weight biomolecules may be used to advantage for the perfusion-dependent NMR enhancement of organs such as the lungs and heart [14]. An additional benefit of these paramagnetic biomolecules is that the conjugation to a large molecular weight species improves the proton relaxation enhancement of these conjugates. The effect is attributed to changes in correlation times [8].

Covalent modification of serum albumins with nitroxyl spin labeling reagents have been reported previously [15–19] as part of investigations of protein conformational mobilities using ESR. The multiple free amino groups in serum albumin are covalently linked with various nitroxyls, the efficacy of the interaction depending on both the solubility of the nitroxyl species in the aqueous solution of BSA and the pH. Although several methods for the activation of carboxyl groups in nitroxyl molecules are available, such as, the use of acyl halides, symmetric or asymmetric anhydrides, and carbodiimides [20–22], use of active esters is preferable because of relative ease of preparation, characterization and storage, and ready solubility in aqueous media. Additionally, the

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by-products of active esters in their conjugation with serum albumins are easily removable. Hence, in the present study two active esters **3** and **5** are employed to form bovine serum albumin (BSA)-nitroxyl conjugates.

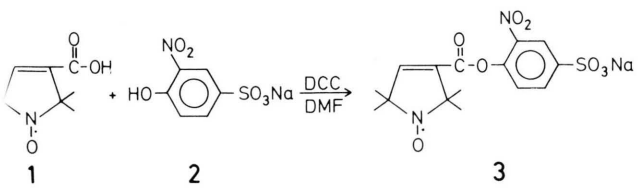
Additionally, paramagnetic metallo complex conjugates of BSA were synthesized as possibly superior alternatives for intravascular NMR contrast agents. The use of complexon moieties, such as CDTA, DTPA, and TTHA resulting in chelates with large stability constants [23, 24] can either diminish or eliminate the toxicity associated with metal cations [25]. In the present study, gadolinium^{+3} was chosen as the most suitable ion since it possesses a high magnetic moment, seven unpaired electrons, a relatively long electronic relaxation time, and a labile hydration sphere.

In anticipation of strong proton relaxation effects, conjugation of BSA with the gadolinium chelates containing polyamino carboxylate functions, including CDTA (**7**), DTPA (**11**) and TTHA (**14**) are now explored. Furthermore, the incorporation of the nitroxyl moiety into such chelates prior to conjugation with BSA, and the interaction of the resulting spin labeled BSA complexons **17, 18** and **19** with gadolinium to give spin labeled BSA complexon-metal ion conjugates **20, 21** and **22,** respectively, could provide useful probes for the ESR analysis of pharmacokinetics.

Recently, we reported the spin labeled chelating agents and their gadolinium complexes as possible contrast enhancers in NMR imaging [26]. We now report the synthesis and proton relaxation effectiveness of spin labeled BSA **4** and **6,** spin labeled BSA complexons **17, 18** and **19,** and their gadolinium complexes **20, 21** and **22** as agents with potential applications for *in vivo* NMR contrast enhancement.

Results and Discussion

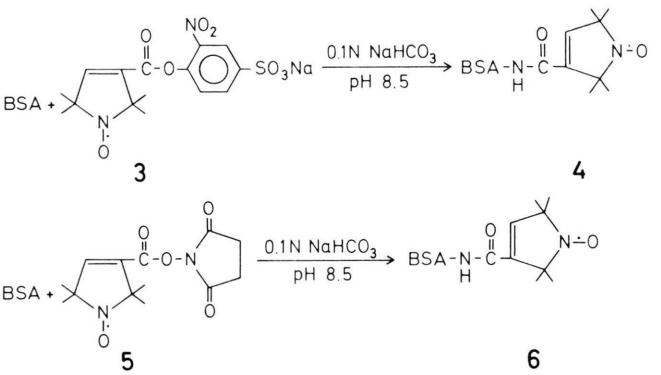
The transfer reagent **3** was prepared in 90% yield by reacting **1** and **2** in the presence of dicyclohexylcarbodiimide (DCC) in accordance with the Scheme I.



1 2 3

cheme I.

The transfer reagent **5** was synthesized according to the literature method [27]. Bovine Serum Albumin was spin labeled with **3** and **5** to give **4** and **6,** respectively (Scheme II). In the spin labeling of the BSA



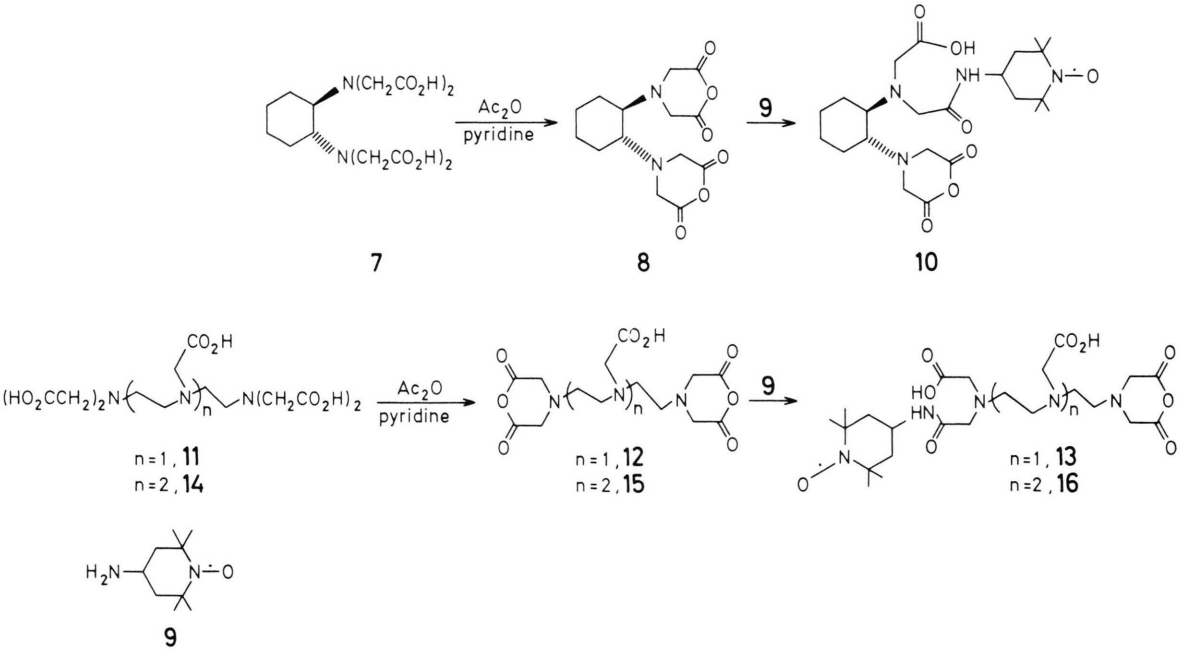
Scheme II.

using water soluble transfer reagents **3** and **5** (Scheme II), the substitution on the protein was achieved 1) without the use of organic solvents and 2) with a better control over the number of substituents per protein molecule than that obtained with water insoluble active esters [20–22]. Further, the use of water soluble active esters simplifies the purifications process since all reaction by-products, such as, the 1-hydroxy-2-nitrobenzene-4-sulfonic acid (by using **3**) or N-hydroxysuccinimide (by using **5**), and the unreacted excess of the transfer reagents **3** and **5,** and the nitroxyl carboxylates are water soluble and, hence, were readily removed by exhaustive dialysis against a 0.1 N ammonium hydroxide solution. Lyophilization of the dialysate yielded the nitroxyl spin labeled proteins **4** and **6** as dry powders.

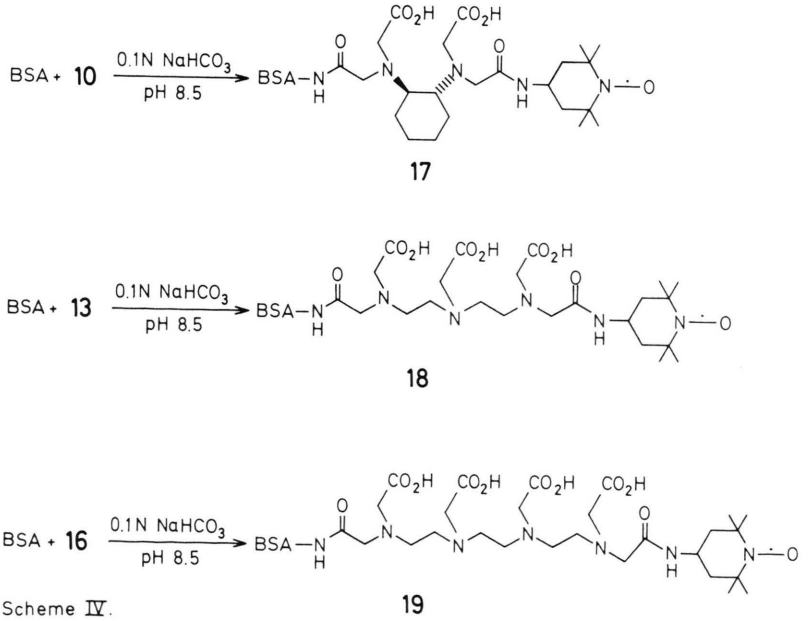
The dianhydrides **8** and **12** were prepared according to the literature method [5]. Dianhydride **15** was analogously prepared by an adaptation of the literature procedure [5] (Scheme III). These dianhydrides were reacted [26, 28] with the 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**9**) under anhydrous conditions to give the corresponding monoradical monoanhydrides **10, 13** and **16** in yields ranging between 58 percent and 65 percent (Scheme III). These monoanhydrides (**10, 13** and **16**) were used for the spin labeling of the BSA (Scheme IV) under similar experimental conditions, as those used for the preparation of **4** and **6.** Extensive dialysis of the crude products followed by lyophilization of the dialysate

yielded the spin labeled BSA complexons **17, 18** and **19** as dry powders. In both spin labeling procedures (Scheme II and IV) the reaction medium was maintained at a pH of 8.5 in order to assure the condensation of amino groups of BSA with the respective nitroxyl derivatives to form amide bonds between the amino functionalities and the carboxylate moiety of the nitroxyl radical.

The spin labeled BSA complexons were then conjugated with the gadolinium ion by the methods analogous to that reported in the literature [20, 29]. Thus, compounds **17, 18** and **19** were reacted with

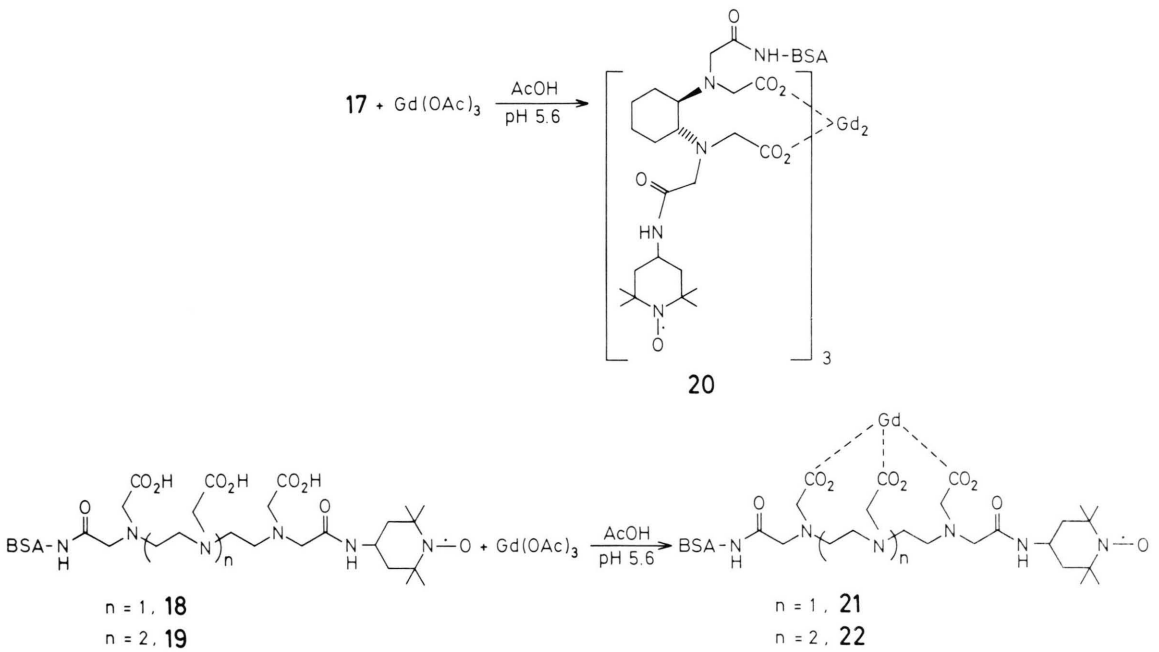


Scheme III.



Scheme IV.

gadolinium acetate at pH 5.6 in the presence of 0.1 M aqueous acetic acid at a metal ion to protein ratio of 30:1 to yield crude products **20, 21** and **22,** respectively (Scheme V). Exhaustive dialysis of these crude products against a 0.1 N ammonium hydroxide solution removed all adventitiously bound gadolinium metal ions, and successive lyophilization gave dry powders of **20, 21** and **22.** In order to establish any possible direct bonding between the gadolinium ions and the BSA, a control experiment was conducted by mixing gadolinium acetate and native untreated BSA under experimental conditions similar to those which were used in the preparation of conjugates **20, 21** and **22.** Extensive dialysis of the mixture, followed by lyophilization gave a solid possessing relaxation times T_1 (T_1 = 2095 m sec) and T_2 (T_2 = 178 m sec) which were similar to those found for the deionized water (T_1 = 2150 m sec, T_2 = 182 m sec, Table I). This result indicates that the gadolinium ions are either not bound or bound re-



Scheme V.

Table I. Spin-lattice (T_1) and spin-spin (T_2) relaxation times and the relaxivities of spin labeled BSA and their gadolinium complexes[a].

Compound[b]	Relaxation Times[c] (m sec) T_1	T_2	Relaxivity[d] (liter $mmol^{-1} \cdot sec^{-1}$)	Spin count (spins/molecule)
4	350	58	40	9.6
6	240	20	39	9.5
17	210	19	48	9.4
18	208	19	49	9.6
19	205	18	50	9.4
20	39	15	369	9.4
21	33	10	414	9.5
22	31	8	432	9.5
Gd-DTPA[e]			4.52–6.74[f]	

[a] The concentration of each sample was 100 μM. Each sample, except **6,** was dissolved in deionized water. Compound **6** was dissolved in a 250 mMolar ammonium bicarbonate solution; [b] EPR spectra were obtained using 100 μMolar solutions; [c] relaxation times T_1 and T_2 were measured at 24 °C. For deionized water T_1 = 2150 m sec, T_2 = 182 m sec; [d] slopes of $1/T_1$ *versus* concentration plots (50–200 μM); [e] in clinical tests Refs. [6, 7]; [f] see Ref. [35].

versibly to the BSA. On the basis of this result it can be further concluded that in conjugates **20, 21** and **22** the gadolinium ion can only be bound to the BSA protein *via* the carboxylate moieties of the bifunctional chelates CDTA, DTPA and TTHA and not directly to BSA.

The number of spins per molecule of the BSA protein were calculated [30] using the double integrals of the first derivatives of the EPR spectra of the spin labeled BSA derivatives **4, 6, 17, 18** and **19** and for their gadolinium conjugates **20, 21** and **22.** Thus, a spin count of between 9–10 spins/molecule of BSA was obtained for these spin labels **4, 6, 17, 18** and **19** and for their gadolinium conjugates **20, 21** and **22.**

The relaxation times T_1 and T_2 of the spin labeled BSA derivatives **4, 6, 17, 18, 19, 20, 21** and **22** were measured at 100 μM concentrations in deionized water, except for **6** which was measured in ammonium bicarbonate solution. The results are shown in Table I. The relaxivities for these samples were obtained using the relaxation times (T_1) measured at 50, 100, 150 and 200 μM concentrations of these compounds and calculating the slopes from $1/T_1$ *versus* concentration plots. The slopes of these plots were calculated using the least square method. The relaxivity values are shown in Table I.

The values of the relaxation times T_1 and T_2 are lower for the spin labeled BSA complexon gadolinium conjugates **20, 21** and **22** than those of the spin labeled BSA complexons **17, 18** and **19** and these values are, in turn, lower than the values for the spin labeled BSA derivatives **4** and **6** containing only the nitroxyl moieties. This result is attributed to the presence of the gadolinium ion in the conjugates **20, 21** and **22** as compared either to the spin labeled BSA complexons without gadolinium **17, 18** and **19** or to the spin labeled derivatives **4** and **6.** The larger the chelate group, the slower the tumbling rate, and, hence, the lower the relaxation values. The increase in the T_1 and T_2 values for these spin labeled derivatives is in the following order: **22** < **21** < **20** < **19, 18, 17** < **6** < **4.** The lower the relaxation values, the better the relaxivity. The T_1 relaxivity values of the spin labeled BSA conjugates are, therefore, in the following order: **22** > **21** > **20** > **19, 18, 17** > **4** > **6** (Table I).

In conclusion, the spin labeled BSA-complexon-gadolinium conjugates **20, 21** and **22** contain gadolinium, which is a very efficient relaxation enhancer, and a nitroxyl marker which could be useful in monitoring pharmacokinetics by ESR in biological systems [31]. Hence, compounds **20, 21** and **22** appear to have superior relaxation characteristics as potential NMR contrast enhancing agents. Additional tests of stabilities, toxicities, pharmacokinetics and *in vivo* effectiveness will serve to optimize the choice of a candidate compound for further development.

Experimental

Materials and methods

All chemicals were of the finest quality available commercially. Bovine Serum Albumin (BSA) was purchased from Sigma Chemical Co. Solvents were dried using standard procedures [32]. Authentic samples of sodium 4-hydroxy-3-nitrobenzene sulfonate [33] (**2**), N-succinimidiyl-2,2,5,5-tetramethyl-1-oxyl-3-pyrroline-3-carboxylate [27] (**5**) and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl [34] (**9**) were prepared by literature methods.

Analytical procedures

All melting points were obtained with a Thomas-Hoover capillary melting point apparatus using a calibrated thermometer and are uncorrected. For purity control TLC precoated sheets, silica gel 60 F_{254} were used. The microanalyses were performed on a Perkin-Elmer 240 C Elemental Analyzer. IR Spectra were recorded on a Nicolet MX-1 FT/IR Spectrophotometer. Mass spectra were obtained on a Hewlett-Packard mass spectrometer, model 5985 GS, using a direct insertion probe, a source pressure of 2×10^{-7} torr, and either methane as a reactant gas for chemical ionization or electron impact. Therefore, either the $M^+ + 1$ or M^+ values are reported. The EPR spectra of either 10^{-4} M aqueous solutions of the nitroxyl radicals or 100 μM aqueous solutions of the BSA spin labels were recorded in a capillary on a Varian E-115 EPR Spectrometer. The spin count of the BSA spin labels was calculated [30] using the double integrals of the first derivatives of the EPR spectra of the corresponding BSA spin labels.

The relaxation times (Table I) of 100 μM solutions of the BSA spin labels were measured at 24 °C using saturation recovery for T_1 and spin echo for T_2 methods on a Praxis pulsed NMR analyzer model PR-103, 10.7 MHz, 0.251 Tesla, the Praxis Corp. San Antonio, Texas, USA. The relaxivities (Table I) of these BSA spin labels were calculated from the slopes of the plots of $1/T_1$ versus concentrations (50–200 μM). The values of the slopes were computed using least square methods.

Preparation of sodium 4-(2',2',5',5'-tetramethyl-1'-oxyl-3'-pyrroline-3'-carbonyloxy)-3-nitrobenzenesulfonate (**3**)

To a stirred solution of 3-carboxy-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl (**1,** 0.552 g, 3.0 mmol) and sodium 4-hydroxy-3-nitrobenzenesulfonate [27] (**2,** 0.723 g, 3.0 mmol) in dimethylformamide (5 ml) was added in one portion dicyclohexylcarbodiimide (0.618 g, 3.0 mmol) at 5 °C. The reaction mixture was stirred for 5 h at 20 °C and the separated solid, N,N'-dicyclohexylurea, was collected by filtration. The solid was successively washed with dimethylformamide (2 ml), tetrahydrofuran (2×2 ml) and ethyl ether (2 ml). The combined washings and filtrate were concentrated on a rotating evaporator at 50 °C/1 torr. The remaining yellow oil was dissolved in ethanol (4 ml) and the resultant solution diluted with ethyl ether (20 ml) whereby a solid material precipitated. Collection of this solid by filtration and washing with ethyl ether (3×5 ml) gave 1.1 g (90%) of **3** which gradually decomposes, without melting, above 250 °C.

MS (EI, 15 eV): *m/e* = 407 (M^+, 50%), 392 (M^+-15, 42%), 279 (M^+-128, 48%), 167 (M^+-240, 70%), 149 (M^+-258, 100%).

IR (KBr) *v:* 1046, 1160, 1176, 1190, 1229, 1350, 1536, 1753, 2938, 2980, 3015 cm^{-1}.

$C_{15}H_{16}N_2O_8SNa$ (407.353) [%]
Calcd C 44.23 H 3.96 N 6.87,
Found C 44.60 H 3.44 N 6.89.

Spin labeling of Bovine Serum Albumin (BSA) using spin labeled transfer reagents **3** *and* **5.** *Preparation of* **4** *and* **6**

Bovine Serum Albumin (250 mg, 4 μmol) was dissolved over a period of 10–15 min in a 0.1 N sodium bicarbonate solution (7.5 ml) and to this solution was added either the active ester **3** (204 mg, 0.5 mmol) or the active ester **5** (141 mg, 0.5 mmol). The reaction mixture was diluted to 30–40 ml using deionized water. The pH of the solution was adjusted to 8.5 with a 0.1 N sodium hydroxide solution. The solution containing the active ester **5** was stirred at ambient temperature for 24 h for complete dissolution of the active ester **5.** The solution was kept in a refrigerator at 4 °C for 24 h adjusting the pH to 8.5 with a 0.1 N sodium hydroxide solution (2–5 ml). The solution was then exhaustively dialyzed in a 0.1 N ammonium hydroxide solution (1000 ml) for 24 h. The eluant was replaced with a freshly prepared 0.1 N ammonium hydroxide solution (1000 ml) every 4 h. The eluant was periodically checked for the presence of free nitroxyl radicals by ESR spectroscopy. After 24 h the eluant contained no free nitroxyl radicals. After the dialysis the solution was lyophilized to give 190 mg of the yellow colored spin labeled BSA derivative **4** using the active ester **3,** and 190 mg of the white colored spin labeled BSA derivative **6** using the active ester **5.**

Preparation of **8, 12** *and triethylenetetraamine-hexaacetic acid dianhydride* **15**

The dianhydrides **8** and **12** were prepared according to the literature method [26]. Compound **15** was analogously prepared by an adaptation of the literature procedure [26].

A mixture of **14** (1.731 g, 3.5 mmol), acetic anhydride (3 ml) and pyridine (2 ml) was stirred at 45–48 °C for 2 h, and at 24 °C for 6 h. To the reaction mixture was added dry ether (15 ml). The precipitated white solid was collected by filtration and washed with ether. Recrystallization of the solid from dimethylformamide : ether (1:1, v/v) gave 1.30 g (75%) of **15** m.p. 195–198 °C (dec.).

MS (CI): *m/e* = 299 (M^+-159, 15%), 286 (M^+-172, 32%), 245 (M^+-213, 22%), 217 (M^+-241, 100%), 203 (M^+-255, 72%), 160 (M^+-298, 46%), 132 (M^+-326, 36%).

$C_{18}H_{26}N_4O_{10}$ (458.424) [%]
Calcd C 47.16 H 5.72 N 12.22,
Found C 47.22 H 5.79 N 12.42.

Preparation of the monoanhydride monoradicals of the chelating agents **10, 13** *and* **16.** *General procedure*

A solution of the corresponding dianhydride **8** (0.838 g, 2.7 mmol) or **12** (0.965 g, 2.7 mmol) or **15** (1.238 g, 2.7 mmol) and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**9,** 0.47 g, 2.7 mmol) in dry dimethylformamide (64 ml) and dry benzene (4 ml) was heated at 65–70 °C for 3 h. The solvent was removed on a rotating evaporator at 40 °C/20 torr. The residue was purified by flash chromatography on silica gel 60 using methylene chloride : methanol (2:1, v/v) as eluants. Removal of the solvents at 25 °C/20 torr from the appropriate eluates gave 0.890 g (65%) of **10** m.p. 139–141 °C (dec.) or 0.886 g (62%) of **13** m.p. 182–184 °C (dec.) or 0.988 g (58%) of **16** m.p. 191–193 °C (dec.).

$C_{23}H_{37}N_4O_7$ (481.57) [%]
Calcd C 57.36 H 7.74 N 11.63,
Found C 57.62 H 7.91 N 11.76.

$C_{23}H_{38}N_5O_9$ (528.583) [%]
Calcd C 52.26 H 7.25 N 13.25,
Found C 52.54 H 7.14 N 13.10.

$C_{27}H_{45}N_6O_{11}$ (629.688) [%]
Calcd C 51.50 H 7.20 N 13.35,
Found C 51.71 H 7.09 N 13.25.

Spin labeling of BSA using spin labeled chelating agents. Preparation of **17, 18** *and* **19**

Bovine Serum Albumin (250 mg, 4 μmol) was dissolved over a period of 10–15 min in a 0.1 N sodium bicarbonate solution (7.5 ml). To the solution the appropriate spin labeled chelating agent either **10** (241 mg, 0.5 mmol) or **13** (264 mg, 0.5 mmol) or **16** (315 mg, 0.5 mmol) was added. The reaction mixture was diluted to 30–40 ml using deionized water. The pH of the solution was adjusted to 8.5 with a 0.1 N sodium hydroxide solution. The solution was stirred at ambient temperature for 8 h, adjusting the pH to 8.5 every 2–3 h, and then kept in a refrigerator at 4 °C for 12 h. The pH of the solution was adjusted once again to 8.5 with a 0.1 N sodium hydroxide solution (2–5 ml) and the solution was exhaustively dialyzed in a 0.1 N ammonium hydroxide solution (1000 ml) for 24 h. During the dialysis, the eluant was replaced every 3–4 h with a freshly prepared 0.1 N ammonium hydroxide solution (1000 ml). The eluant was periodically checked for the presence of nitroxyl radicals by the ESR spectroscopy. After 24 h the eluant contained no free nitroxyl radicals. After the dialysis the sample was lyophilized to give 200 mg of a white colored spin labeled BSA **17** or **18** or **19,** respectively.

Preparation of gadolinium complexes **20, 21** *and* **22** *using spin labeled bovine serum albumin chelating agents* **17, 18** *and* **19.** *General procedure*

To a solution of spin labeled bovine serum albumin chelating agent either **17** or **18** or **19** (25 μmol) in 0.1 M aqueous acetic acid (10 ml) was added gadolinium acetate tetrahydrate (750 μmol) at a metal ion:protein ratio of 30:1. The pH of the solution was adjusted to 5.6 using a 1.0 M aqueous acetic acid solution. After standing for 30 min at 24 °C, the solution was exhaustively dialyzed against a 0.1 M ammonium hydroxide solution (pH ~8) for 48 h. The solution was then lyophilized for 24 h to give 1.4 g of solid compounds **20, 21** and **22,** respectively.

A control experiment using gadolinium acetate and bovine serum albumin

To a solution of the bovine serum albumin (25 μmol) in 0.1 M aqueous acetic acid (10 ml) was added gadolinium acetate tetrahydrate (750 μmol) at a metal ion:protein ratio of 30:1. The pH of the solution was adjusted to 5.6 using a 1.0 M aqueous acetic acid solution. After standing for 30 min at 24 °C, the solution was exhaustively dialyzed against a 0.1 M ammonium hydroxide solution (pH ~8) for 48 h. The solution was then lyophilized for 24 h to give 1.45 g of a solid. The relaxation times T_1 and T_2 of a 100 μmolar aqueous solution of this solid resulted in similar values (T_1 = 2095 m sec, T_2 = 178 m sec) as those obtained for the deionized water (T_1 = 2150 m sec, T_2 = 182 m sec). This result indicated that the gadolinium is either not bound or reversibly bound to the native BSA protein.

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