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Target Visualization by MRI Using the Avidin/Biotin Amplification Route: Synthesis and Testing of a Biotin–Gd-DOTA Monoamide Trimer**

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Abstract: To design efficient targeting strategies in magnetic resonance (MR) molecular imaging applications, the formation of supramolecular adducts between (strept)avidin ((S)Av) and tribiotinylated Gd-DOTA-monoamide complexes (DOTA = 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) was explored. Two compounds based on the trivalent core of tris(2-aminoethyl)amine each containing three biotin molecules and one (**L1**) or three (**L2**) DOTA-monoamide (DOTAMA) ligands were synthesized. In these tribiotinylated derivatives the biotins are spaced far enough apart to allow the formation of the supramolecular adduct with the protein and to host the chelating units in between the (S)Av layers. Size exclusion HPLC analyses indicated complete formation of very high molecular weight polymers (>2 MDa) with (S)Av in solution. A ^{1}H NMR spectroscopy relaxometric study on the obtained polymeric adducts showed a marked increase of the relaxivity at 35–40 MHz as a consequence of the lengthening of the tumbling time due to the formation of Gd-chelates/(S)Av polymers. The most efficient Gd_3**L2**/(S)Av polymeric system was used for a test in cell cultures. The target is represented by a neural cell adhesion molecule (NCAM), which is overexpressed in Kaposi's sarcoma cells and tumor endothelial cells (TEC) and that is efficiently recognized by a biotinylated tetrameric peptide (C3d-Bio). In vitro experiments showed that only cells incubated with both C3d-Bio and Gd_3**L2**/SAv polymer were hyperintense with respect to the control. Relaxation rates of cell pellets incubated with Gd_3**L2**/SAv alone were not significantly different from the untreated cells demonstrating the absence of a specific binding.

Keywords: gadolinium · imaging agents · magnetic resonance imaging · NMR spectroscopy · supramolecular chemistry

Introduction

Much attention is currently devoted to elucidating the cellular and molecular mechanisms of diseases through the use of noninvasive, high resolution, in vivo imaging techniques.[1]

In vivo molecular imaging procedures aim at providing a visual representation of molecules and molecular events that have key roles in pathological processes. Among the various imaging modalities, magnetic resonance imaging (MRI) yields high-resolution 3D maps of the body (or parts of it) by exploiting the differences in relaxation times of tissue water protons in the anatomical region of interest. The addition of exogenous contrast agents (CA), such as paramagnetic Gd^{III} complexes or iron oxide nanoparticles, greatly increases MRI tissue differentiation and adds further physiological information to the superb anatomical resolution of MR images.[2] The specificity of a CA can be pursued by conjugating the contrast enhancing unit to a well-defined targeting molecule to bind specific molecular epitopes that

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[**] MRI = magnetic resonance imaging; DOTA = 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid.

act as a kind of signature of the pathological process.[3] However, whereas MRI can provide high image resolution (50 μm^2 in vivo) when compared with optical and nuclear techniques, it suffers from a limited sensitivity of its probes.[1] Therefore, targeted CAs require site-specific accumulation capabilities to allow the visualization of targets that are present at subnanomolar concentration.[2,3] At the imaging fields of 0.5–1.5 T, one of the possible routes to attain high relaxivity Gd-based CAs deals with the elongation of the molecular reorientational time of the Gd^{III} complex by forming noncovalent adducts between the complex and slowly tumbling systems.[4] Thus, macromolecular carriers loaded with a large number of Gd^{III} complexes have been widely studied.[5,6]

Considering that (strept)avidin ((S)Av) shows an extremely high affinity for biotin ($K_a = 1 \times 10^{15}\,M^{-1}$), the recognition process of the (S)Av–biotin pair has been widely used as an amplification tool for both in vitro bioassays and in vivo medical applications.[7] D. S. Wilbur and co-workers have extensively explored this approach by the development of many molecules involved in the polymerization and/or cross-linking of (S)Av. The focus of the studies was the investigation of antibody conjugates with recombinant streptavidin in tumor pretargeting protocols, a method for amplifying the amount of radioactivity delivered to cancer cells.[8] Many derivatives have been synthesized by Wilbur and coworkers containing two and three biotin moieties with varying distances between the carbon atoms of the biotin amide.[9] Only tris-biotinylated compounds completely polymerized in SAv solution and similar results were obtained in the surface-bound SAv cross-linking experiment.

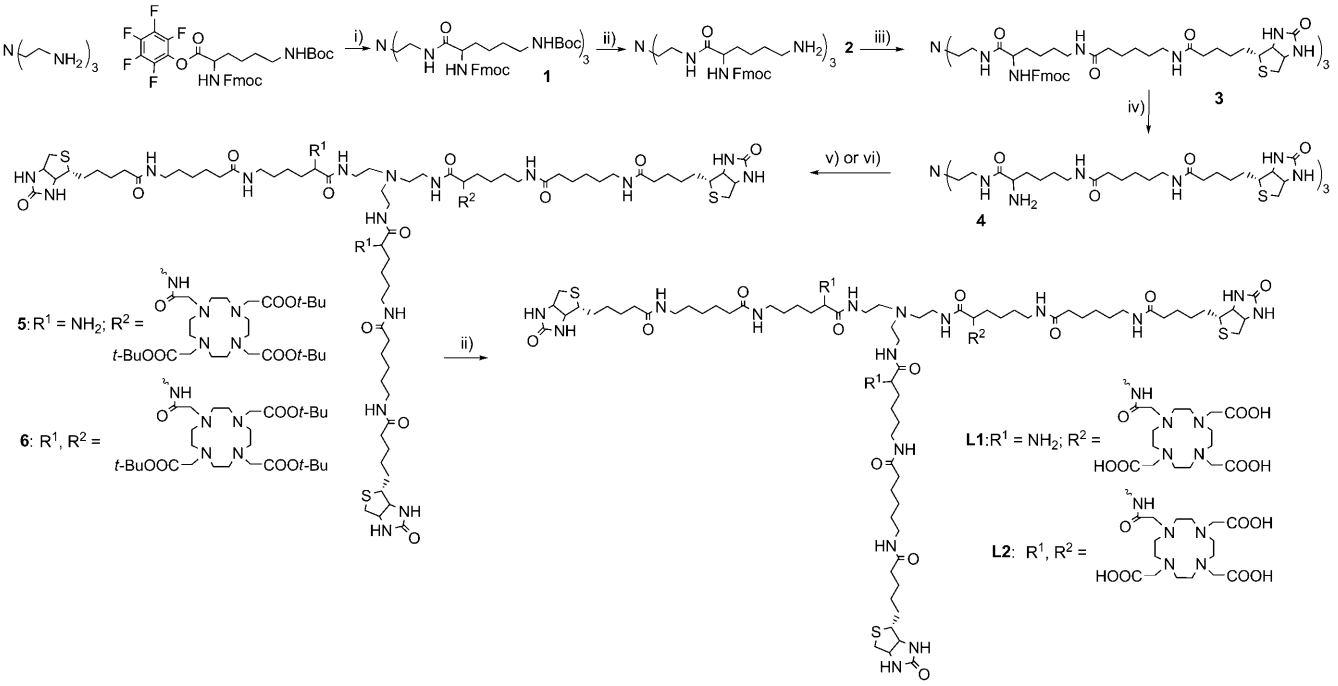
We have recently tackled the issue of the low sensitivity of MRI targeting experiments by exploiting the amplification properties of supramolecular adducts formed between avidin and biotinylated Gd^{III} chelates.[10] Gd-DTPA-like complexes (DPTA = diethylenetriamine pentaacetic acid) containing one and two biotin residues were synthesized and their adducts with avidin were tested in vitro. Evidence for the occurrence of multilayered adducts in which the bis-biotinylated Gd^{III} complex bridged adjacent avidin molecules was obtained. However, the use of dimeric biotin ligands strongly limited the polymerization reaction resulting in a complex mixture of oligomers of different Gd payload. To fulfill the stereochemical requirements for an efficient build up of large polymeric adducts with (S)Av, the synthesis of tribiotinylated linkers with one (**L1**) and three (**L2**) DOTA-monoamide (DOTAMA) ligands attached has been pursued. Size exclusion chromatography was employed to assess the formation of the polymeric Gd**L1**/(S)Av and Gd_3**L2**/(S)Av adducts. The Gd_3**L2**/(S)Av polymeric system was further used to validate an in vitro MR molecular imaging protocol based on targeting neural cell adhesion molecules (NCAM) in Kaposi's sarcoma cells and tumor endothelial cells (TEC)[11] pretargeted with a suitable biotinylated peptide.

Results and Discussion

Probe design: Since the dimensions of Av are $55 \times 55 \times 41$ Å and the biotin binding site is about 9 Å below the surface of the protein, linkers smaller than 15 Å are not long enough to allow the second biotin molecule to reach the binding site on another Av molecule. Only linkers in which the distance between biotin moieties (between amide carbon atoms) is longer than 20 Å, result in the intermolecular binding of the second biotin.[9] Moreover, in the case of well-spaced bis-biotinylated compounds the formation of long chain polymers is, in part, hampered by the occurrence of intramolecular linkages, as previously shown in the case of biotin–DTPA dimers.[10] Therefore, molecules with three biotin moieties have to be designed to allow the formation of polymeric chains. Furthermore, we have to take into account not only that two of the three biotin moieties in tribiotinylated compounds would bind to the same protein molecule, but also the space needed for the insertion of three Gd^{III}-DOTAMA complexes. Therefore, we designed two tribiotinylated probes **L1** and **L2** starting from the trifunctional core of tris(2-aminoethyl)amine (TREN). The linker contains amide bonds and short aliphatic chains to yield a distance between the biotin amide carbon atoms that enables the polymerization with (S)Av, as well as providing enough space for the accommodation of Gd-DOTAMA units in between two adjacent proteins (Scheme 1).

Synthesis: For both **L1** and **L2**, the synthesis started from the coupling of TREN with the pentafluorophenyl activated ester of the orthogonally protected α-Fmoc-ε-Boc lysine (Fmoc = 9-fluorenylmethoxycarbonyl, Boc = *tert*-butoxycarbonyl) in CH_2Cl_2. Product **1** was isolated in high yields by simple filtration and then the ε-Boc protecting group was removed by acid cleavage to obtain **2** in almost quantitative yield. At this point the three free amino groups of lysine were treated with the carboxylic acid of D-biotinamido hexanoic acid by using classic coupling reagents, such as HATU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate) and DIPEA (*N*,*N*-diisopropylethylamine) in DMF. After pouring the mixture into iced water, product **3** was collected by centrifugation as a solid powder, pure enough for the subsequent Fmoc cleavage with a solution of piperidine (20% in DMF). The addition of diethyl ether to the mixture allowed the precipitation of the intermediate **4**, which was isolated in 95% yield.

Compound **4** is the starting material for both **L1** and **L2**, since the α-NH_2 groups could react with the free carboxylic acid group of a suitable protected chelator that, after deprotection, would be used for the complexation of Gd^{3+} ions. Considering the thermodynamic and kinetic stabilities of Gd^{III} complexes,[2] the DOTA-monoamide (DOTAMA) ligand (DOTA = 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) has been chosen as a chelator for the paramagnetic metal ion (Figure 1). Thus, the tris(*tert*-butyl)-protected DOTA ligand (DOTA(O^tBu)$_3$) was coupled to **4** to obtain a DOTAMA coordinating cage after cleavage of the



Scheme 1. i) Tris(2-aminoethyl)amine, Fmoc-Lys(Boc)-OPfp, CH_2Cl_2, 48 h; ii) CF_3COOH, CH_2Cl_2, 18 h; iii) D-biotinamido hexanoic acid, HATU, DIPEA, DMF, 24 h; iv) 20% piperidine in DMF, 3 h; v) DOTA($OtBu$)$_3$, HATU, DIPEA, DMF, 3 h; vi) DOTA($OtBu$)$_3$, HATU, DIPEA, DMF, (((8 h.

tert-butyl groups. Unexpectedly, only the product with one DOTAMA ligand attached to the TREN-Lys-biotin scaffold was obtained (**5**). Attempts with higher ratios between DOTA($OtBu$)$_3$ and **4** did not result in an increase in the number of macrocyclic moieties linked to the scaffold. We surmised that the attachment of only one DOTAMA chelate to the TREN-Lys-biotin scaffold may be due to steric hindrance at the core of the molecule and/or the difficulty of the acid functional group on the DOTA($OtBu$)$_3$ derivative to access the amino groups present on the linker backbone. In fact, hydrogen bond interactions in the TREN-Lys-biotin scaffold may cause intramolecular folding, thus masking the two amino groups and blocking further coupling. With the aim of disrupting intramolecular hydrogen bonds and promoting the complete coupling reaction, the conjugation of DOTA($OtBu$)$_3$ by using the same procedure as described above (i.e., HATU, DIPEA in DMF) has been carried out under power ultrasound (US) irradiation (20.2 kHz, 60 W). As demonstrated in a recent paper,[12] the use of power ultrasound allows the formation of the desired product providing enough energy to dissociate these intramolecular hydrogen bonds. Thus, by means of US activation, compound **6** was obtained in high yields (80%). This finding is then consistent with the view that two of the three amino groups of **4** are almost completely protected towards coupling to DOTA($OtBu$)$_3$ and only the use of US allows their deprotection. Finally, **L1** and **L2**, after cleavage of *tert*-butyl esters with trifluoroacetic acid and precipitation with diethyl ether, were both obtained as a white powder.

Formation of polymeric (S)Av adducts: Gd-DOTAMA tribiotinylated Gd**L1** and Gd_3**L2** were evaluated for their ability to polymerize (S)Av in aqueous solution. Following the procedure reported by Wilbur et al.,[9] to get polymerization to occur, varying molar quantities of tribiotinylated probes were mixed with solutions containing (S)Av in phosphate-buffered saline (PBS), incubated at room temperature, and then evaluated by size exclusion chromatography. In the present study we have used a Superose 6 preparative grade (GE Healthcare life science) home-packed column that has a separation range between 5 kDa and 5 MDa and an exclusion limit of 40 MDa (based on protein assays). The chromatograms for mixtures containing molar quantities of Av/Gd**L1** varying from 1:0 to 1:1.5 and of Av/Gd_3**L2** varying from 1:0 to 1:1 are shown in Figure 1A and B, respectively. The chromatograms of the mixtures (Gd**L1**/Av and Gd_3**L2**/Av) clearly indicate the presence of high molecular weight species characterized by a chromatographic peak with a shorter retention time than Av. In fact, the addition of Gd**L1** or Gd_3**L2** to Av solution causes a decrease of the intensity of the Av peak (retention time, 45.7 min) accompanied by the increase of a new peak characterized by a shorter retention time (20.5 min) that was attributed to very high molecular weight Gd**L1**/Av or Gd_3**L2**/Av polymers.

Since Dextran Blue (MW$\approx$2.0 MDa) is the highest molecular weight standard available that could be measured on Superose 6 (retention time, 26.7 min), a molecular weight higher than 2 MDa can be reasonably estimated for Gd**L1**/Av and Gd_3**L2**/Av polymers. The difference between Gd**L1**/Av and Gd_3**L2**/Av systems relies on the Av/biotin trimer

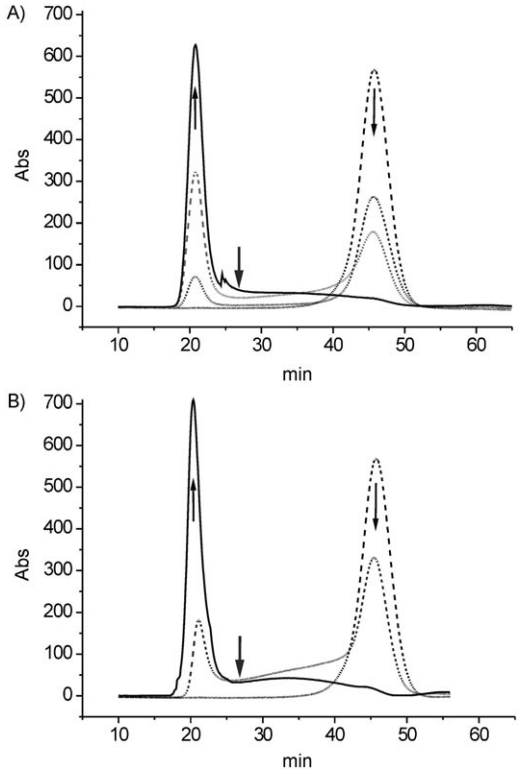


Figure 1. Size exclusion chromatograms of Av (black dashed line) and reaction mixtures after mixing Av with tris-biotin Gd**L1** (A) and Gd_3**L2** (B) at RT for 10 min. Complete formation of the polymeric adducts (retention time = 20.5 min for both) is shown by the chromatograms (black line) at different Gd-chelate/Av ratios (1.5:1 for Gd**L1** and 1:1 for Gd_3**L2**). Grey dashed lines report the chromatograms related to intermediate Gd-chelate/Av ratios. The arrow at 26.7 min indicates the retention time of Dextran Blue (MW ≈ 2 MDa). See Chromatography in the Experimental Section for details of the HPLC equipment and conditions used.

molar ratio needed to complete the polymerization. In fact, in the case of Gd_3**L2** the polymer was fully formed at a 1:1 molar ratio, whereas in the case of Gd**L1**, at a 1:1 molar ratio the peak relating to free Av was still present and only after a further addition of Gd**L1** linker (final ratio 1:1.5) was a complete disappearance of the Av peak observed to indicate the occurrence of complete polymerization.

This different behavior could be ascribed to the different steric hindrance of Gd**L1** and Gd_3**L2**. Avidin, in fact, has four biotin binding sites symmetrically distributed in two pairs on opposite sides of the protein. Tentatively, as schematically depicted in Figure 2, one may envisage that two molecules of Gd**L1**, less hindered than Gd_3**L2**, occupy two binding sites on the same protein side, giving a supramolecule characterized by a ratio of linker/protein higher than one. On the contrary, the steric hindrance of the three DOTAMA units in Gd_3**L2** prevents this possibility, giving a supramolecular polymer characterized by a ratio of linker/protein equal to one.

Since for in vivo applications SAv has to be used instead of Av, an analogous evaluation for the ability of Gd**L1** and Gd_3**L2** to polymerize SAv in aqueous solution was carried

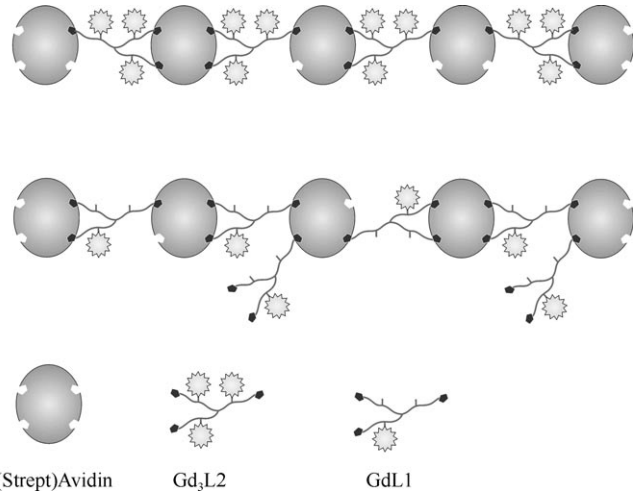


Figure 2. Schematic representation of the formation of polymeric Gd**L1**/(S)Av and Gd_3**L2**/(S)Av adducts showing the different Gd-chelate/(S)Av ratios necessary to obtain complete polymerization.

out. The formation of polymers at the same molar ratios as used in the case of Av confirmed that the use of SAv does not alter the ability of these tribiotinylated systems to form polymeric adducts.

Relaxometric characterization of the avidin/streptavidin adducts: The relaxivities (i.e., the proton relaxation enhancements of water protons in the presence of the paramagnetic complex at 1 mM concentration) of Gd**L1** and Gd_3**L2**, measured at 20 MHz and 298 K, are 7.0 and 8.5 $mM^{-1}s^{-1}$, respectively. As expected, Gd_3**L2** displays higher relaxation rates (and a relaxivity per molecule of 25.5 $mM^{-1}s^{-1}$), because of the presence of three Gd-DOTAMA units per molecule. The exchange rate ($k_{ex} = 1/\tau_M$) of the inner-sphere water molecule was determined by measuring the transverse relaxation time of ^{17}O-nuclei of solvent water molecules as a function of temperature and by fitting the obtained data to the values calculated on the basis of Swift–Connick theory.[13] Gd**L1** and Gd_3**L2** displayed a τ_M value, at 298 K, of about 0.94 and 0.92 μs , respectively, in agreement with values previously reported for a number of analogous Gd^{III} complexes with variously substituted DOTAMA ligands.[14] Having independently measured τ_M, the parameters affecting the observed proton relaxivity can be assessed by analyzing the dependence of the ^1H-water relaxation rates as a function of the applied magnetic field ($1/T_1$ NMR dispersion profile) (Figure 3). The best fit of the data to the values calculated on the basis of the Solomon–Bloembergen–Morgan equations[15] (for the inner-sphere contribution) and of Freed's equation[16] (for the outer-sphere contribution) indicated that the increase in relaxivity with respect to a typical Gd-DOTAMA complex ($r_{1p} = 4.6$ $mM^{-1}s^{-1}$)[14] is due to the lengthening of τ_R (Table 1) as a consequence of the increased molecular size (Gd**L1**: MW = 2092.8; Gd_3**L2**: MW = 3180.1). The supramolecular adducts with Av were formed by mixing stoichiometric amounts of the protein with a

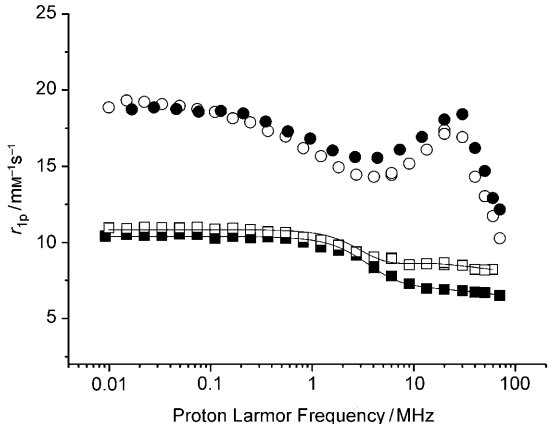


Figure 3. $1/T_1$ ^{1}H NMRD spectroscopy profile of Gd**L1** (■), Gd_3**L2** (□), Gd**L1**/Av (●), and Gd_3**L2**/Av (○) (pH 7; $T=25$°C). The solid line through the data points was calculated with the parameters reported in Table 1.

0.2 mM solution of the Gd complex for 30 min at room temperature in PBS. The adduct formation was followed by recording NMR dispersion (NMRD) profiles that showed a marked increase of the relaxivity peak centered at 35–40 MHz. This increase is the consequence of the further lengthening of the tumbling time due to the aggregation of different Av units. The NMRD profiles of the two adducts (Gd**L1**/Av and Gd_3**L2**/Av) appear quite similar indicating that the two polymers are essentially the same size, according to HPLC results. The relaxivities at 20 MHz, 298 K are 18.1 and 17.4 mM^{-1}s^{-1} for Gd**L1**/Av and Gd_3**L2**/Av, respectively. By using the polymer molecular weight extrapolated from HPLC data (>2 MDa), it is possible to calculate that each Av polymer contains at least 30 tribiotinylated complexes. Therefore, a relaxivity (20 MHz and 298 K) >550 for Gd**L1**/Av and >1600 for Gd_3**L2**/Av can be estimated. On this basis the more efficient Gd_3**L2** complex was selected for further cell targeting and MRI visualization tests.

Furthermore, the serum stability of Gd_3**L2**/Av was investigated to assess the polymer degradation by the enzyme biotinidase (25 nM in human serum) or by other proteases present in blood.[8,17] Since the relaxivity of the Gd_3**L2**/Av adduct is more than two times higher than the relaxivity of free Gd_3**L2**, the degradation of the adduct can be followed by measuring the proton relaxation rate (R_{1obs}) of the solution. For this purpose, the R_{1obs} of solutions containing Gd_3**L2**/Av (0.5 mM protein concentration) were measured after dilution 1:10 with human serum. The measured relaxation rate (3.1 s^{-1} at 20 MHz) remained unchanged during the monitored period of 24 h after serum addition. We conclude that the stability of the Gd_3**L2**/Av adduct is high enough to prevent its degradation when injected into the blood stream.

Table 1. Selected best fit parameters obtained from the analysis of the $1/T_1$ NMRD and ^{17}O NMR spectroscopy profiles of the Gd**L1** and Gd_3**L2** complexes.

Complex	Δ^2 [s^{-2}/10^{19}]	τ_v [ps]	τ_r [ps]	τ_m [μs]	ΔH_m [kJ mol^{-1}]	ΔH_v [kJ mol^{-1}]
Gd**L1**	2.2[a]	27[a]	160[a]	0.94[b]	17[b]	10[b]
Gd_3**L2**	1.8[a]	38[a]	240[a]	0.92[b]	17[b]	10[b]

[a] Best-fit parameters obtained from the analysis of the NMRD spectroscopy profile (298 K) by considering one inner sphere water molecule ($q=1$) the protons of which are at an average metal distance of 3.0 Å. [b] Best-fit parameters obtained from the analysis of the ^{17}O NMR spectroscopy profile by considering the temperature dependence of ^{17}O-R_{2p} for a 12 mM solution of Gd**L1** and Gd_3**L2** by using a Gd–^{17}O scalar coupling constant of -3.8×10^6 rad s^{-1} and a Gd–^{17}O distance of 2.5 Å.

In vitro MRI of tumor-derived endothelial and Kaposi's sarcoma cells: Recently, the expression of the embryonic form of neural cell adhesion molecules (NCAM) by Kaposi's cells and by tumor endothelial cells (TEC) has been reported. [11] NCAM is an adhesion molecule structurally belonging to the immunoglobulin super family that mediates cell to cell interactions.[18] Moreover, NCAM is expressed by several solid tumors and some types of leukemia.[19] By exploiting a high affinity NCAM-binding peptide (C3d)[20] we have recently shown the possibility of targeting NCAM in vivo in neoformed vessels.[21] C3d is a dendrimeric peptide composed of four monomers coupled to a lysine backbone (C3 peptide = ASKKPKRNIKA),[20] that is easily biotinylated in solid phase at the four terminal NH_2 groups by the standard biotin/HATU/DIPEA method to obtain C3d-Bio in good yields.[21] Here, we investigated the binding of the Gd_3**L2**/SAv polymer to the tumor cell surface, incubated previously with an excess of C3d-Bio that interacts selectively with the NCAM receptors. For cell experiments SAv was used instead of Av because SAv has the advantage of much lower nonspecific binding, since it lacks any carbohydrate modification and has a near-neutral isoelectric point (pI). The two-step binding experiment was carried out at 20°C with or without the preincubation (20 min) of a 20-fold excess of C3d-Bio on both Kaposi's and TEC cells. After washing, the cells were incubated for 20 min at 20°C in the presence of the Gd_3**L2**/SAv polymer. As shown in Figure 4, only cells incubated with both C3d-Bio and Gd_3**L2**/SAv were hyperintense with respect to the unlabeled control cells. Figure 4 shows that the proton relaxation rate of cell pellets incubated with Gd_3**L2**/SAv alone is not significantly different with respect to the control, thus demonstrating the absence of specific binding of the supramolecular probe for tumor cells. The amount of Gd bound to cells was measured by inductively coupled plasma mass spectrometry (ICP-MS; see Table 2) and varies according to MRI results. The increased amount of Gd found after the preincubation with C3d-Bio indicates that free biotin binding sites remain available on the Gd_3**L2**/SAv polymer to promote the binding with the biotinylated peptide.

Conclusion

In principle, the supramolecular adduct Gd_3**L2**/SAv imaging probe has the advantage of being very versatile, since it may

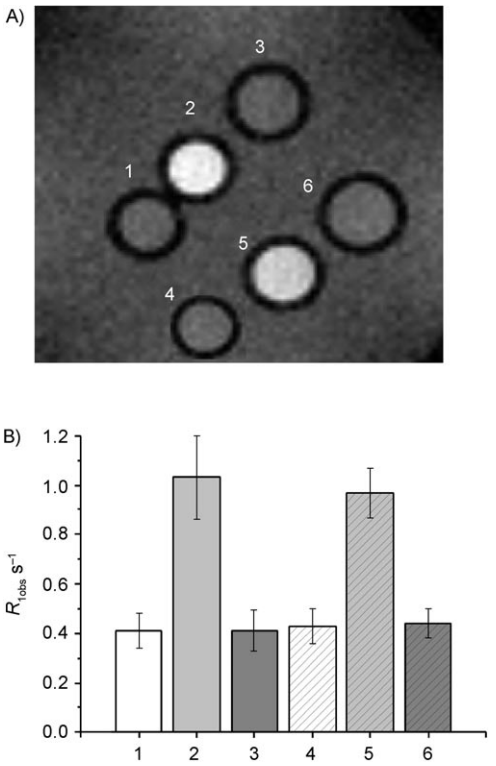


Figure 4. A) T_1-weighted spin echo MR image (7 T), of an agar phantom containing Kaposi's (**1**, **2**, **3**) and TEC (**4**, **5**, **6**) cells incubated (20 min at 20 °C) with Gd_3**L2**/SAv (30 μM) in the absence (**3** and **6**) and in the presence (**2** and **5**) of C3d-Bio (600 μM) and unlabeled cells (**1** and **2**). B) Relaxation rates measured at 7 T on the corresponding samples.

Table 2. The amount of Gd bound to cells (measured by ICP-MS) expressed as nmol of Gd/mg of protein.

	Cells incubated with C3d-Bio + Gd_3**L2**/SAv	Cells incubated with Gd_3**L2**/SAv
Kaposi's	5.5	0.5
TEC	3	0.8

be used in different diagnostic, as well as therapeutic modalities. In fact, the polymeric adduct may be built up by mixing Gd_3**L2** with variable amounts of other biotinylated compounds containing optical or positron emission tomography/single photon emission computed tomography (PET/SPECT) probes or prodrugs in suitable amounts to fit with the sensitivity of the different imaging modalities and the pharmacological doses, respectively. Furthermore, since only three of the biotin binding sites are occupied by the three Gd_3**L2** biotins, it would be possible to exploit the Gd_3**L2**/SAv polymer for the targeted delivery of biotinylated drugs and/or other probes, obtaining a dual probe for imaging and therapy and/or for different imaging modalities. Finally, it must be noted that Gd_3**L2** can be accumulated at different pathological sites by simply changing the biotinylated unit responsible for the specific targeting (i.e., peptide, vitamin, or amino acid).

Experimental Section

All chemicals were purchased from Sigma–Aldrich and were used without purification unless otherwise stated. NMR spectra were recorded on a on a JEOL Eclipse Plus 400 and a Bruker Avance 600 (operating at 9.4 and 14 T, respectively). The cavitating tube (20.2 kHz) used in the present work was developed by authors in collaboration with Danacamerini s.a.s. (Torino).[22] ESI mass spectra were recorded on a Waters Micromass ZQ. Analytical and preparative HPLC-MS were carried out on Waters FractionLynx autopurification system equipped with a Waters 2996 diode array and Waters Micromass ZQ (ESCI ionization mode) detectors. DOTA(O*t*Bu)$_3$ was prepared following a reported procedure.[23] NMRD profiles were measured at 25 °C over a continuum of magnetic field strengths from 0.00024 to 0.47 T (corresponding to 0.01–20 MHz proton Larmor Frequency) on a Stelar field-cycling relaxometer (Stelar, Mede, Italy), under complete computer control with an absolute uncertainty of 1 %. Data points from 0.47 T (20 MHz) to 1.7 T (70 MHz) were collected on a Spinmaster spectrometer (Stelar, Mede, Italy) working at variable field.

Synthesis of *N,N′,N″*-tris(3-aza-5-amidofluorenylmethyl-10-*N*-*tert*-butoxycarbonyl-4-oxodecane)amine (1): Tris(2-aminoethyl)amine (472.3 mg, 3.23 mmol), dissolved in anhydrous CH_2Cl_2 (20 cm^3), was added dropwise in 1 h to a stirred solution of Fmoc-Lys(Boc)-OPfp (OPfp = pentafluorophenyl ester) (6.77 g, 10.7 mmol) in CH_2Cl_2 (40 cm^3). The mixture was left stirring under inert atmosphere at RT for 48 h. During this time a precipitate was formed, which was collected by filtration and dried under vacuum. By reducing the volume of the solvent and by freezing the resulting organic solution another batch of precipitate was collected. The total amount of product obtained was 3.96 g (2.65 mmol, 82 % yield). ^{1}H NMR ($CDCl_3$): δ = 7.76 (d, J = 7.2 Hz, Ar*H*, 6H), 7.55–7.49 (m, Ar*H*, 6H), 7.31 (t, J = 7.2 Hz, Ar*H*, 6H), 7.22 (t, J = 7.2 Hz, Ar*H*, 6H), 6.05 (br s, CON*H*, 3H), 4.34–4.24 (m, CH_2-fluorenyl, 6H), 4.06 (t, J = 6.8 Hz, C*H* fluorenyl, 3H), 3.27, 2.98, 2.83, 2.45–2.38 (m, CH_2N, 24H), 1.75, 1.61, 1.45–1.30 (m, CH_2 aliphatic chain, 18H), 1.44 ppm (s, (CH_3)$_3$, 9H); ^{13}C NMR ($CDCl_3$): δ = 174.3 (*C*ONH, 3C), 158.5 (O*C*=ONH, 3C), 143.8, 141.3, 127.8, 127.1, 125.1, 124.9, 120.0 (fluorenyl *C*, 36C), 79.5 (*C*(CH$_3$)$_3$, 3C), 67.5 (*C*H$_2$OCO, 3C), 54.9, 54.6, 46.9, 40.3 (N*C*H$_2$, 12C), 38.6 (*C*HCH$_2$O, 3C), 32.2, 29.5, 22.8 (*C*H$_2$ aliphatic chain, 12C), 28.5 ppm (C-(*C*H$_3$)$_3$, 3C); MS (ESI): m/z: calcd for $C_{84}H_{109}N_{10}O_{15}$: 1497.81 [$M$+H$^+$]; found: 1498.25.

Synthesis of *N,N′,N″*-tris(3-aza-5-amidofluorenylmethyl-10-amino-4-oxodecane)amine (2): Neat trifluoroacetic acid (20 cm^3) was slowly added to a solution of **1** (2.0 g, 1.34 mmol) in CH_2Cl_2 (20 cm^3), cooled in an ice bath. The solution was stirred for 3 h at RT and then evaporated. The residue was again dissolved in CH_2Cl_2 (20 cm^3) and treated with trifluoroacetic acid (20 cm^3). The solution was stirred for 16 h, then it was evaporated and Et_2O (20 cm^3) was added to the oily residue to give a white solid, which was filtered and washed with Et_2O (5 × 30 cm^3) and dried in vacuum (1.55 g, 1.29 mmol, 96 % yield). ^{1}H NMR (CD_3OD): δ = 7.75 (d, J = 7.2 Hz, Ar*H*, 6H), 7.61–7.55 (m, Ar*H*, 6H), 7.35 (t, J = 7.2 Hz, Ar*H*, 6H), 7.25 (t, J = 7.2 Hz, Ar*H*, 6H), 4.38, 4.30–4.21 (m, CH_2-fluorenyl, 6H), 4.13 (t, J = 6.8 Hz, C*H* fluorenyl, 3H), 4.06–4.00 (m, NHC*H*CO, 3H) 3.62, 3.50–3.44 (m, CH_2NH$_2$, 6H), 3.42–3.32 (m, CONHCH_2, 6H), 2.84 (t, J = 7.0 Hz, NCH_2, 6H), 1.76, 1.61, 1.45–1.30 ppm (m, CH_2 aliphatic chain, 18H); ^{13}C NMR (CD_3OD): δ = 174.9 (*C*ONH, 3C), 157.3 (O*C*=ONH, 3C), 143.9, 141.3, 127.5, 126.8, 124.8, 119.7 (fluorenyl *C*, 36C), 66.8 (*C*H$_2$OCO), 55.2, 53.7 (N*C*H$_2$, 6C); 39.1 (*C*H$_2$NH$_2$, 3C), 34.5, 30.8, 26.8, 22.6 ppm (*C*H$_2$ aliphatic chain, 12C); MS (ESI): m/z: calcd for $C_{69}H_{85}N_{10}O_9$: 1197.65 [M+H$^+$] (M); found: 1197.81.

Synthesis of *N,N′,N″*-tris(3,10-diaza-5-amidofluorenylmethyl-17-*N*-biotinamide-4,11-dioxoeptadecane)amine (3): Solid HATU (740 mg, 1.96 mmol) and DIPEA (680 μL, 3.92 mmol) were added to a suspension of D-Biotinamido hexanoic acid (0.64 g, 1.79 mmol) in DMF (20 cm^3) and kept at 0 °C. While maintaining the mixture at 0 °C under vigorous stirring, a solution of **2** (0.67 g, 0.56 mmol) in DMF (15 cm^3) was added dropwise in 1 h. The gel suspension obtained after 24 h was dropped in a beaker containing iced water to form an off-white precipitate, which was collected by centrifugation. The solid was then washed with H_2O and

acetone (3×30 cm^3) and dried under vacuum (1.06 g, 0.47 mmol, 84% yield). ^{1}H NMR (DMSO): $\delta=7.87$ (d, $J=7.2$ Hz, Ar*H*, 6H), 7.78 (s, CON*H*, 3H), 7.73–7.64 (m, Ar*H* and CON*H*, 9H), 7.40 (t, $J=7.2$ Hz, Ar*H*, 6H), 7.30 (t, $J=7.2$ Hz, Ar*H*, 6H), 6.40 and 6.34 (s, N*H*CON*H* biotin, 6H), 4.33–4.24 (m, C*H*NH biotin, 6H), 4.23–4.16 (m, CH_2-fluorenyl, 6H), 4.14–4.06 (m, C*H* fluorenyl, 3H), 3.95–3.86 (m, CON*H*, 3H), 3.14–3.04 (m, NHCH_2CH_2, C*H*NH-Fmoc and C*H*S, 12H), 3.02–2.92 (m, CONHCH_2, 12H), 2.80, 2.57 (dd, $J=12.4$ and 5.0 Hz, CH_2S, 6H), 2.48 (t, $J=7.0$ Hz, NCH_2, 6H), 2.06–1.96 (m, CH_2CO, 12H), 1.66–1.12 ppm (m, CH_2 aliphatic chain, 54H); ^{13}C NMR (DMSO): $\delta=174.5$, 172.7, 172.4 (carboxyamide groups, 9*C*), 163.2 (NH*C*ONH biotin, 3C); 157.3 (O*C*=ONH, 3C), 143.5, 141.3, 128.2, 127.6, 125.8, 120.6 (fluorenyl *C*, 36C), 66.2, 61.6, 59.8, 56.0, 47.3, 38.9, 36.0, 35.8, 29.6, 29.3, 28.8, 28.6, 26.8, 25.9, 25.6, 23.5 ppm; MS (ESI): *m*/*z*: calculated for $C_{117}H_{161}N_{19}O_{18}S_3$: 1108.07 [$M$+2H$^+$,z=2]; found 1108.10.

Synthesis of *N,N′,N″*-tris(3,10-diaza-5-amino-17-*N*-biotinamide-4,11-dioxoeptadecane)amine (4): Compound **3** (150 mg, 0.066 mmol) was treated with a piperidine solution (20% in DMF, 10 cm^3) and the resulting mixture was left stirring at room temperature for 3 h. Et_2O was added to the resulting gel suspension to obtain a white precipitate, which was collected and dried under vacuum (101 mg, 0.063 mmol, 95.4% yield). ^{1}H NMR (D_2O): $\delta=4.65$, 4.67–4.62 (m, $J=12.4$ and 5.0 Hz, C*H*NH biotin, 6H), 3.42–3.30 (m, NHCH_2CH_2, C*H*NH$_2$ and C*H*S 12H), 3.24–3.15 (m, CONHCH_2, 12H), 3.03, 2.81 (dd, $J=12.4$ and 5.0 Hz, CH_2S, 6H), 2.73 (t, $J=7.0$ Hz, NCH_2, 6H), 2.32–2.23 (m, CH_2CO, 12H), 1.84–1.28 ppm (m, CH_2 aliphatic chain, 54H); ^{13}C NMR (D_2O): $\delta=177.7$, 176.6, 176.5 (carboxamido groups, 9C), 165.5 (NH*C*ONH biotin, 3C), 62.4, 60.5, 55.7, 54.9, 52.8, 44.8, 40.0, 39.4, 36.0, 35.9, 28.6, 28.3, 28.0, 25.9, 25.6, 25.4, 22.7, 22.5, 21.8 ppm; MS (ESI): *m*/*z*: calcd for $C_{72}H_{13}0N_{19}O_{12}S_3$: 1548.93 [$M$+H$^+$]; found 1549.15.

Synthesis of *N*-[3,10-diaza-5-[1-carboxyamidomethyl-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane)-17-*N*-biotinamide-4,11-dioxoeptadecane-*N′,N″*-bis(3,10-diaza-5-amino-17-*N*-biotinamide-4,11-dioxoeptadecane)] amine (5): DOTA(tBuO)$_3$ (172.5 mg, 0.3 mmol), HATU (117 mg, 0.31 mmol), and DIPEA (104 μL, 0.6 mmol) were dissolved in anhydrous DMF (10 mL) while the system was stirred under an inert atmosphere at 0°C. After 15 min the ice bath was removed and the mixture was left stirring at RT for 2 h. At this point, a solution of **4** (240.5 mg, 0.15 mmol) in DMF (5 mL) was added dropwise in 30 min and the mixture was stirred at RT for 16 h. The DMF was then evaporated and the residue was taken up in CH_2Cl_2 (15 mL) and washed with H_2O (10 mL), then with a 5% $NaHCO_3$/water solution (10 mL), and finally with H_2O (10 mL). The organic solution was then dried over Na_2SO_4, filtered, evaporated, and dried under vacuum to obtain a pale yellow oil (272.5 mg, 0.12 mmol, 80% yield). ^{1}H NMR ($CDCl_3$): $\delta=7.80$ (s, CON*H*, 4H), 6.47 and 6.40 (s, N*H*CON*H* biotin, 6H), 4.34–4.28, 4.16–4.12 (m, C*H*NH biotin, 6H), 4.21–4.16 (m, C*H*-DOTAMA, 1H), 3.40 (s, CH_2COOtBu and CH_2CONH, 8H), 3.10–2.99 (br m, macrocyclic CH$_2$, 16H), 3.05 (br s, NHCH_2CH_2, C*H*NH$_2$ and C*H*S, 11H), 3.00–2.90 (m, CONHCH_2, 12H), 2.80, 2.57 (dd, $J=12.4$ and 5.0 Hz, CH_2S, 6H), 2.45 (br s, NCH_2, 6H), 2.07–1.98 (m, CH_2CO, 12H), 1.65–1.15 (m, CH_2 aliphatic chain, 54H), 1.44, 1.42 ppm (s, C(CH_3)$_3$, 27H); ^{13}C NMR ($CDCl_3$): $\delta=173.5$, 172.9, 172.5, 172.1, 171.6 (carboxyl and carboxamido groups, 13C), 163.2 (NH*C*ONH biotin, 3C), 81.6, 81.2, 65.8, 61.6, 59.8, 56.4, 56.0, 55.6, 52.5–49 (br m, NCH$_2$ ring), 38.9, 36.0, 35.7, 29.6, 29.3, 28.8, 28.5, 28.0, 26.6, 26.0, 25.8, 25.5, 23.5 ppm; MS (MALDI-TOF): *m*/*z*: calcd for: $C_{100}H_{180}N_{23}O_{19}S_3$: 1052.15 [$M$+2H$^+$, z=2]; found: 1052.26.

Synthesis of *N*-[3,10-diaza-5-[1-carboxyamidomethyl-4,7,10-triscarboxylmethyl-1,4,7,10-tetraazacyclododecane)-17-*N*-biotinamide-4,11-dioxoeptadecane-*N′,N″*-bis(3,10-diaza-5-amino-17-*N*-biotinamide-4,11-dioxoeptadecane)]amine (L1): Neat trifluoroacetic acid (5 cm^3) was slowly added to a solution of **5** (272.5 mg, 0.12 mmol) in CH_2Cl_2 (5 cm^3) cooled in an ice bath. The solution was stirred for 3 h at RT and evaporated. The residue was again dissolved in CH_2Cl_2 (5 cm^3) and treated with trifluoroacetic acid (5 cm^3). The solution was stirred for 16 h, then it was evaporated and Et_2O (10 cm^3) was added to the oily residue to give a white solid, which was filtered and washed with Et_2O (3×10 cm^3) and dried in vacuum (204 mg, 0.097 mmol, 81% yield). ^{1}H NMR (D_2O): $\delta=4.53$, 4.39–4.30 (m, C*H*NH biotin, 6H), 4.18–4.12 (m, C*H*-DOTAMA, 1H), 3.61 (br s, CH_2COOH and CH_2CONH, 8H), 3.38–3.05 (br m, macrocyclic CH$_2$, 16H), 3.26–3.15 (m, NHCH_2CH_2, C*H*NH$_2$, C*H*S, 11H), 3.12–3.03 (m, CONHCH_2, 12H), 2.92, 2.69 (dd, $J=12.4$ and 5.0 Hz, CH_2S, 6H), 2.18–2.10 (m, CH_2CO, 12H), 1.82–1.12 ppm (m, CH_2 aliphatic chain, 54H); ^{13}C NMR (D_2O): $\delta=177.0$, 176.5, 176.2, 175.5, 171.6 (carboxyl and carboxamido groups 13C), 165.2 (NH*C*ONH biotin, 3C), 62.0, 60.2, 55.7, 55,2, 54.0, 53.2, 52–48 (br m, NCH$_2$ ring), 39.8, 39.4, 39.1, 38.9, 38.7, 35.8, 35.6, 35.3, 30.5, 30.2, 28.2, 27.9, 27.6, 25.8, 25.6, 25.3, 25.1, 24.9, 22.8, 21.6 ppm; MS (ESI): *m*/*z*: calculated for $C_{88}H_{156}N_{23}O_{19}S_3$: 1935.11 [$M$+H$^+$]; found: 1935.07.

Synthesis of *N,N′,N″*-tris(3,10-diaza-5-[1-carboxyamidomethyl-4,7,10-tris-(tert-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane]-17-*N*-biotinamide-4,11-dioxoeptadecane)amine (6): Solid HATU (75.5 mg, 0.2 mmol) and DIPEA (70 μl, 0.4 mmol) were added to a suspension of **4** (101 mg, 0.063 mmol) in DMF (20 cm^3) kept at 0°C. While maintaining the mixture at 0°C, a solution of DOTA(tBuO)$_3$ (115 mg g, 0.2 mmol) in DMF (15 cm^3) was added dropwise. The mixture was kept in a high power US bath (20.2 kHz, 60 W) for 8 h. The gel suspension obtained was dropped in a beaker containing iced H_2O to form a slight yellow precipitate, which was collected by centrifugation. The solid was then washed with H_2O (2×20 cm^3) and dried in vacuum (165 mg, 0.05 mmol, 80.2% yield). ^{1}H NMR ($CDCl_3$): $\delta=7.75$ (s, CON*H*, 6H), 6.45 and 6.39 (s, N*H*CON*H* biotin, 6H), 4.35–4.28, 4.27–4.20 (m, C*H*NH biotin, 6H), 4.16–4.08 (m, C*H*-DOTAMA, 3H), 3.34 (s, CH_2COOtBu and CH_2CONH, 24H), 3.10–2.96 (br m, macrocyclic CH$_2$, 48H), 3.10–3.00 (m, NHCH_2CH_2 and C*H*S, 9H), 2.99–2.91 (m, CONHCH_2, 12H), 2.82, 2.58 (dd, $J=12.4$ and 5.0 Hz, CH_2S, 6H), 2.44 (br s, NCH_2, 6H), 2.09–2.00 (m, CH_2CO, 12H), 1.62–1.18 (m, CH_2 aliphatic chain, 54H), 1.44, 1.42 ppm (s, C(CH_3)$_3$, 81H); ^{13}C NMR ($CDCl_3$): $\delta=173.0$, 172.7, 172.5, 172.2, 171.8 (carboxyl and carboxamido groups, 21C), 163.2 (NH*C*ONH biotin, 3C), 81.7, 81.3, 65.5, 61.6, 59.8, 56.2, 56.0, 55.8, 53–48 (br m, NCH$_2$ ring), 38.9, 35.8, 35.6, 29.5, 29.3, 28.7, 28.5, 28.1, 26.8, 26.0, 25.7, 25.4, 23.5 ppm; MS (MALDI-TOF): *m*/*z*: calcd for: $C_{156}H_{281}N_{31}O_{33}S_3$: 1606.52 [$M$+2H$^+$, z=2]; found: 1606.49.

Synthesis of *N,N′,N″*-tris(3,10-diaza-5-[1-carboxyamidomethyl-4,7,10-triscarboxylmethyl-1,4,7,10-tetraazacyclododecane]-17-*N*-biotinamide-4,11-dioxoeptadecane)amine (L2): Neat trifluoroacetic acid (5 cm^3) was slowly added to a solution of **6** (165 mg, 0.05 mmol) in CH_2Cl_2 (5 cm^3) cooled in an ice bath. The solution was stirred for 3 h at RT and then evaporated. The residue was again dissolved in CH_2Cl_2 (5 cm^3) and treated with trifluoroacetic acid (5 cm^3). The solution was stirred for 16 h, then it was evaporated and Et_2O (10 cm^3) was added to the oily residue to give a white solid, which was filtered and washed with Et_2O (5×10 cm^3) and dried in vacuum (113 mg, 0.042 mmol, 84% yield). ^{1}H NMR (D_2O): $\delta=$ 4.54–4.48, 4.37–4.30 (m, C*H*NH biotin, 6H), 4.17–4.10 (m, C*H*-DOTAMA, 3H), 3.60–3.50 (br m, CH_2COOH and CH_2CONH, 24H), 3.34–3.02 (br m, macrocyclic CH$_2$, 48H), 3.26–3.18 (m, NHCH_2CH_2 and C*H*S, 9H), 3.09–3.02 (m, CONHCH_2, 12H), 2.90, 2.68 (dd, $J=12.4$ and 5.0 Hz, CH_2S, 6H), 2.18–2.10 (m, CH_2CO, 12H), 1.74–1.21 ppm (m, CH_2 aliphatic chain, 54H); ^{13}C NMR (D_2O): $\delta=176.5$, 175.5, 173.0, 171.0 (carboxyl and carboxamido groups, 21C), 163.2 (NH*C*ONH biotin, 3C), 66.0, 62.0, 60.2, 55.5, 54.0, 53.5, 52–48 (br m, NCH$_2$ ring), 39.8, 38.9, 35.8, 35.5, 30.5, 30.1, 28.5, 28.2, 28.6, 25.8, 25.5, 25.1, 23.5 ppm; MS (MALDI-TOF): *m*/*z*: calcd for: $C_{120}H_{209}N_{31}O_{33}S_3$: 1354.24 [$M$+2H$^+$, z=2]; found: 1354.43.

Synthesis of Gd complexes: The Gd**L1** and Gd_3**L2** complexes were prepared by mixing stoichiometric amounts of each ligand and $GdCl_3$ solution at RT. The excess Gd^{3+} ions were precipitated as $Gd(OH)_3$ at basic pH. The amount of residual free Gd^{3+} ion was assessed by the orange xylenol UV method.[24] Gd**L1** and Gd_3**L2** were found to contain less than 0.3% (mol/mol) of residual free Gd^{3+} ion. The overall Gd content was determined by ^{1}H NMR spectroscopy T_1 measurement of the mineralized complex solution (in 6M HCl at 120°C for 16 h). MS (MALDI-TOF) for Gd**L1**: *m*/*z*: calcd for: $C_{88}H_{154}Gd_1N_{23}O_{19}S_3$: 2091.76 [$M$ + H$^+$]; found: 2091.97; for Gd_3**L2**: *m*/*z*: calculated for $C_{120}H_{201}Gd_3N_{31}O_{33}S_3$: 1587.52 [$M$+2H$^+$, z=2]; found 1587.83. The isotopic distributions are consistent with the Gd^{III} complexes.

Chromatography: A 100 μL aliquot of (S)Av (0.5 mg mL^{-1} in PBS) was placed in an Eppendorf vial. Gd**L1** or Gd_3**L2** biotin trimer (10 μL) was added rapidly. After incubating at RT for 10 min, the reaction mixtures were injected onto an Amersham Superose 6 size exclusion HPLC column and eluted by an Amersham Akta Purifier chromatographic system equipped with two pumps, a UV/Vis triple wavelength detector (set at 215, 230, and 254 nm), and online pH, conductivity, and temperature monitors. The column was calibrated with reference standard molecules (blue dextran molecular mass 2,000 kDa, ferritin 440 kDa, concanavalin A 110 kDa, ovalbumin 43 kDa, ribonuclease A 13.7 kDa, and tryptophan 204 Da), by using the same buffer and experimental conditions of the analytical runs. The operative workup was carried out under isocratic conditions at 1.0 mL min^{-1}, by using an aqueous mobile phase containing 50 mM potassium phosphate (pH 6.8), and 300 mM NaCl.

Cell lines: Kaposi's and TEC cells were kindly provided by C. Grange (Department of Internal Medicine, University of Torino). Kaposi's cells were maintained in culture in an RPMI medium with 10% fetal calf serum (FCS). TEC were obtained from renal clear cell type carcinomas as previously described;[25] TEC were maintained in culture in EBM complete medium (Cambrex Bioscience, East Rutherford) supplemented with 10% FCS.

Serum Stability: To asses the stability of Gd_3**L2**/Av to biotinidase and other serum proteases, a solution of Gd_3**L2**/Av (0.5 mM protein concentration) was diluted 10-fold in reconstituted human serum (Seronorm, Sero As, Billingstad, Norway) and incubated at 37 °C for 24 h.

Uptake experiments and MRI analysis: For the in vitro binding experiments, Kaposi's and TEC cells were detached by using ethylenediaminetetraacetic acid (EDTA, 1 mM), washed with PBS, counted in a microcytometer chamber and resuspended in DMEM (2×10^6 in 200 μL DMEM). C3d-Bio peptide and Gd_3**L2**/SAv preformed polymer were sequentially added, in the molar ratio 20:1, (corresponding to concentrations of 600 and 30 μM, respectively) to TEC and Kaposi's cells (at 20 °C), at a time interval between the additions of each component of 20 min. After each binding step, the cells were washed three times with ice cold PBS (5 mL). The extent of a specific binding of Gd_3**L2**/SAv polymer was assessed by repeating the above experiments without the addition of the targeting peptide. After washing, the cells were transferred into glass capillaries placed in an agar phantom for MRI analysis. MRI were acquired on a Bruker Avance 300 spectrometer (7 T) equipped with a Micro 2.5 microimaging probe by using a standard T_1-weighted multislice multiecho sequence (TR/TE/NEX = 200:3.3:16, FOV = 1.2 cm, one slice = 1 mm, in-plane resolution = 94 × 94 μm). T_1 measurements of cells were performed by using a standard saturation recovery sequence. The amount of Gd bound to cells was measured by inductively coupled plasma mass spectrometry (ICP-MS, Element-2, Thermo-Finnigan, Rodano (MI) Italy). Sample digestion was performed with concentrated HNO_3 (70%, 2 mL) under microwave heating (Milestone MicroSYNTH Microwave lab station equipped with an optical fiber temperature control and HPR-1000/6M six position high pressure reactor, Bergamo, Italy). After digestion the volume of each sample was brought to 2 mL with ultrapure water, and the sample was analyzed by ICP-MS. Three replicates of each sample solution were analyzed. Protein concentration of each sample was determined from cell lysates by the Bradford method by using bovine serum albumin as standard.

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