Gadolinium-Based Contrast Agents Targeted to Amyloid Aggregates for the Early Diagnosis of Alzheimer's Disease by MRI

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Author Contributions

C.F., M.P., S.C. and G.B. developed the medicinal chemistry strategy. G.B. and H.B. designed the compounds and performed the chemical syntheses and relaxivity characterisations. A.K. performed the chemical syntheses. S.B. performed the binding study and G.L. carried out the study on BBB crossing. G.B. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Abstract

While important efforts were made in the development of positron emission tomography (PET) tracers for the *in vivo* molecular diagnosis of Alzheimer's disease, very few investigations to develop magnetic resonance imaging (MRI) probes were performed. Here, a new generation of Gd(III)-based contrast agents (CAs) is proposed to detect the amyloid β -protein ($A\beta$) aggregates by MRI, one of the earliest biological hallmarks of the pathology. A building block strategy was used to synthesize a library of 16 CAs to investigate structure-activity relationships (SARs) on physicochemical properties and binding affinity for the $A\beta$ aggregates. Three types of blocks were used to modulate the CA structures: (i) the Gd(III) chelates (Gd(III)-DOTA and Gd(III)-PCTA), (ii) the biovectors (2-arylbenzothiazole, 2-arylbenzoxazole and stilbene derivatives) and (iii) the linkers (neutrals, positives and negatives with several lengths). These investigations revealed unexpected SARs and a difficulty of these probes to cross the blood-brain barrier (BBB). General insights for the development of Gd(III)-based CAs to detect the $A\beta$ aggregates are described.

Keywords

Alzheimer's disease, amyloid, gadolinium, DOTA, Pyridine Containing TriAza (PCTA), magnetic resonance imaging (MRI).

Abbreviations

BAT, bis-amine-bis-thiol; BTA, 2-(4-*N*-methylaminophenyl)benzothiazole; CA, contrast agent; CMC, critic micelle concentration; CV, cone voltage; DIEA, *N,N*-diisopropylethylamine; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; EDCI HCl, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride; FDG, fluorodesoxyglucose; HBTU, *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; IBOX, 2-(4-dimethylaminophenyl)-6-iodobenzoxazole; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; ICP-MS, inductively coupled plasma-mass spectroscopy; IMPY, 6-iodo-2-(4'-dimethylamino)-phenyl-imidazo[1,2-*a*]pyridine; LRMS, low-resolution mass spectroscopy; MAMA, monoamide-monoaminedithiol; MCI, mild cognitive impairment; MION, monocristalline iron oxide nanoparticle; NHS, *N*-hydroxysuccinimide; PCTA, 3,6,9,15-tetraaza bicyclo[9.3.1]-pentadeca-1(15),11,13-triene-3,6,9-triacetic acid; PIB, Pittsburgh Compound-B; r_1 , longitudinal relaxivity; r_2 , transversal relaxivities; SB, stilbene; T_1 , spin-lattice relaxation time; T_2 , spin-spin relaxation time; th-T, thioflavin-T; UBTA, *N,N'*-bis[2-mercapto-2-methylpropyl]2-aminobenzylamine; USPIO, ultrasmall superparamagnetic iron oxide.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder responsible for 60% to 70% of dementia cases. About 6% of the population aged 65 and older suffer from dementia, which represented 36 million people worldwide in 2010 with a total societal cost estimated to be US\$ 604 billion. The number of AD patients is believed to double every 20 years to potentially reach 115 million by 2050.[1] AD has both societal and economical impacts on our society and is expected to become one of the major health care problems for industrialized countries in the future.[2] As a matter of fact, the research for a treatment and an early diagnosis of AD, with *in vivo* imaging of amyloid plaques as the most promising technique,[3] appears today as a priority.[4]

In the last 20 years, the research on AD focused on the development of different therapeutic approaches and diagnostic techniques to detect the pathology at an early stage.[5] However, to date the definitive diagnosis of AD can be performed *post-mortem* only.[6] An early diagnosis of AD would allow not only an intervention with future disease-modifying therapies right from the start of the disease, but also a relevant follow-up of drugs tested.[7] The neuropathological hallmarks of AD are intracellular neurofibrillary tangles, caused by the hyperphosphorylation of tau proteins, and extracellular neuritic plaques, caused by the aggregation of amyloid β -protein ($A\beta$) fibrils, accompanied with neurotransmitter deficits. The "amyloid hypothesis", [8] today accepted by a large part of the scientific community, suggests that $A\beta$ plaques appear in the brain 10 to 20 years before the first clinical symptoms of AD. The $A\beta$ aggregates are believed to be one of the most relevant hallmarks along the AD evolution process.[9-10] *In vivo* methods for

amyloid imaging,[11] together with cerebrospinal fluid biomarkers, as well as functional monitoring of the brain (glucose metabolism with [^{18}F]fluorodesoxyglucose ([^{18}F]FDG) or neurotransmitter activity),[10,12] are expected to have a high potential to specifically diagnose AD patients at a very early stage. During the last 10 years, various molecular imaging radiolabeled tracers have been reported for the detection of amyloid deposits by single-photon emission computed tomography (SPECT) and positron emission tomography (PET) and some are currently assessed on clinical trials.[10,13-15] Florbetapir- ^{18}F has been the first amyloid specific ^{18}F PET tracer approved by the Food and Drug Administration (FDA) in 2012 for adults being evaluated for AD or other cognitive disorders.[16] The detection of amyloid deposits in human with the Pittsburgh Compound-B ([^{11}C]PIB) and Florbetapir- ^{18}F by PET imaging showed to be able to differentiate a population at risk of developing AD from mild cognitive impairment (MCI) patients.[17-19] Interestingly, around 20-40% of asymptomatic controls showed a [^{11}C]PIB retention,[20-24] which suggests that this compound might be an efficient tracer for AD preclinical diagnosis since the major growth of amyloid burden seems to occur at this stage of the disease.[25-27]

The development of probes for the detection of the AD hallmarks by nuclear magnetic resonance imaging (MRI) has been much less successful, mainly due to the lower sensitivity of this technique compared to PET and SPECT. To date, magnetic probes have been studied only at a pre-clinical stage. However, the advantages provided by MRI over radionuclide-based imaging techniques encouraged us to investigate in this direction. Compared to SPECT or PET, MRI technique does not require the injection of radioactive probes, has better resolution (200 μm to 50 μm for clinical and research magnets respectively) and provides anatomic information which

could be relevant for quantifying amyloid deposits and characterising precisely the deposition areas.[28-29] The high resolution achievable by MRI makes the detection of amyloid lesions possible both in humans and animals, which is also critical for the evaluation of new treatments against AD at early stages. Moreover, the lower cost, wider availability and the absence of irradiation of the MRI exams afford important advantages.

Contrary to radionuclide-based imaging techniques such as PET and SPECT, MRI does not allow the direct detection of the CA. Indeed the signal detected by MRI is the relaxation rate enhancement of the spins of the water protons in close proximity to the paramagnetic Gd(III) lanthanide. This complex interaction usually prevents the correlation of the local Gd(III) concentration with the MRI signal variation detected in biological media because of the influence of the surrounding of the water protons on the effect of the Gd(III) lanthanide. Nevertheless, specialists agree that the detection of Gd^{3+} ions at concentration of 1-10 μM in the brain could be achieved by using Gd(III)-based MRI CAs with relaxivity equivalent to commercial MRI CAs (i.e. 3 $mM^{-1}.s^{-1}.Gd^{-1}$) and clinical MRI equipment. However, both the density of the targeted site and the relaxivity of the CA bound to the targeted site influence the local concentration of Gd^{3+} ions required for MRI detection. For instance, S. Aime *et al.* estimate that $4 \pm 1 \times 10^7$ Gd^{3+} ions per cell are required to reach a concentration sufficient to be detected *in vivo*, leading to a local Gd^{3+} ion concentration of 2 μM . [30] C. Corot *et al.* reported a folate receptor targeting Gd(III)-based CA which requires a local Gd^{3+} ion concentration of 0.92 μM to be detected *in vitro* thanks to its high longitudinal relaxivity ($r_1 = 25 mM^{-1}.s^{-1}.Gd^{-1}$, 2.35 T, 37 °C). [31] The density of A β peptides in AD frontal cortex brain was determined at 1-3 μM [32] and the binding stoichiometry of CAs to A β in AD brain is close to 1:1 under saturating

conditions.[33] Considering that a relaxivity enhancement of the CAs ($\sim \times 2$ at 60 MHz, 37 °C) is expected after binding to the amyloid aggregates, thanks to an increase of the rotational correlation time,[34] CAs with relaxivities around $10 \pm 5 \text{ mM}^{-1} \cdot \text{s}^{-1}$ have a great potential to detect amyloid aggregates *in vivo* if the targeted sites can be reached.

Several Gd-based CA have been reported for the detection of amyloid aggregates in mice by MRI. The Gd^{III} diethylenetriaminepentaacetic acid (Gd(III)-DTPA) complex was first attached to the N-terminal of the peptide A β ₁₋₄₀. [35] Amyloid deposits could be detected *in vivo* with 7 T μ MRI and T₂* weighted images after transient opening of the blood-brain barrier (BBB) with mannitol. Several ways to enhance BBB permeability of the probes were investigated further. The introduction of putrescine moieties on the peptide A β ₁₋₄₀[36] and on the truncated derivative A β ₁₋₃₀[37] by modification on glutaric acid and aspartic acid residues were investigated by intravenous injections in APP/PS1 transgenic mice and *ex vivo* detection of the probes. The peptide A β ₁₋₃₀ elongated with 6 lysine residues was attached to the Gd(III)-DTPA chelate and the CA was dissolved in a mannitol containing buffer before intracarotid injection in a Tg2576 mouse and *in vivo* detection with 7 T μ MRI and T₂* weighted images.[38] Even if these structure modifications improved BBB permeability without affecting the binding on amyloid deposits, these probes do not cross the BBB enough to be detected *in vivo* by MRI when adjuvants such as mannitol is not used. Recently, a PIB derivative labeled with Gd(III), ¹¹¹In(III) and Eu(III) was reported.[39] In spite of a low binding affinity for the A β ₁₋₄₀ aggregates ($K_d = 180 \text{ }\mu\text{M}$), the Eu(III) derivative CA showed to successfully label the amyloid deposits in *post-mortem* human brain tissues of AD patients. However, the BBB permeability of the CA was

expected to be insufficient for *in vivo* MRI detection. The solubility and the parameters determining the relaxivity of the Gd(III) derivatives was successfully tuned by modifying the linker between the Gd(III) chelate and the PIB derivative.[34] Iron oxide particles such as monocristalline iron oxide nanoparticles[35] (MIONs) and ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles,[40] labeled with A β ₁₋₄₀ and A β ₁₋₄₂ respectively, have been investigated with similar approaches and were able to bind the amyloid aggregates *in vivo* after injection in APP/PS1 transgenic AD mice with 15% mannitol in phosphate buffer saline (PBS). Similarly, these probes validated the possibility to detect amyloid *in vivo* by MRI but their use was limited by a poor BBB permeability. Introduction of PEG on the surface of A β ₁₋₄₂ modified USPIO significantly improved the BBB permeability of the CA.[41] After intravenous injection of this CA without adjuvant, A β aggregates were clearly detected *ex vivo* (overnight scanning, 7 T μ MRI, T₂* weighted images). However, *in vivo* detection was more uncertain due to the low sensitivity and the high background resulting from blood vessels. To date, there is no efficient MRI CA available to detect the amyloid deposits at the early stage of the disease in a living patient. The few studies describing *in vivo* detection of amyloid deposits by MRI all required a co-injection of the CA with mannitol to transiently open the BBB, which prevents applications in clinical trials. For these reasons, new types of CA need to be developed for clinical use.

Among the large numbers of PET tracers developed in the last decade for the detection of amyloid deposits, some showed to be particularly attractive for further development (Fig. 1). The thioflavin-T (th-T) derivative [¹¹C]PIB (**1**), a carbon-11 radionuclide marker of A β plaques,[33,42] is currently the most widely used among the few PET tracers in clinical

trials.[43-49] This gold-standard amyloid PET tracer is in the phase III clinical trials and is often used as a reference to compare the efficiency of new potent markers of A β plaques.[50] The *N,N'*-dimethylated analog of the [^{11}C]PIB revealed similar binding properties with an inhibition constant (K_i) of 4.4 nM (vs 4.3 nM for [^{11}C]PIB) on A β_{1-40} aggregates (2-(4-*N*-methylaminophenyl)benzothiazole ([^3H]BTA) as competitor).[51] The 2-(4-dimethylaminophenyl)-6-iodobenzoxazole ([^{123}I]IBOX, **2**), a benzoxazole analog of the [^{11}C]PIB labeled with iodine 123, was reported to efficiently bind amyloid aggregates in *post-mortem* brain sections of confirmed AD patients.[52] The affinities of several stilbene derivatives for amyloid aggregates in AD patient brain homogenates were reported[53-54] and the radiopharmaceutical 4-(*N*-methylamino)-4'-hydroxy-*trans*-stilbene (K_d 2.4 nM, [^3H]SB-13, **3**) was selected for further investigations in human.[55]

Fig. 1. Chemical structures of amyloid specific ligands [^{11}C]PIB (**1**), [^{123}I]IBOX (**2**) and [^{11}C]SB-13 (**3**).

The structure of these three radiopharmaceuticals inspired us for the design of the biovectors aiming to target the CAs on the amyloid deposits. These low MW chromophores contrast with the long peptide chains previously required to target the MRI CAs. The new generation of CAs investigated was expected to have a different biodistribution profile than those previously developed and cross the BBB. The carbon carrying the hydroxyl function or the iodide atom was selected as the most suitable anchor to graft the imaging moiety according to the previously reported structure-activity relationship (SAR) studies on th-T[56] and stilbene[57] derivatives. Structurally similar probes carrying the metal ^{99m}Tc have been described in the literature for SPECT imaging of amyloid aggregates (Fig. 2). A modified *N,N'*-bis[2-mercapto-2-methylpropyl]2-aminobenzylamine (UBTA) ^{99m}Tc complex grafted to a diphenyl derivative biovector (**4**) showed to bind the amyloid on brain slices from transgenic mouse APP/PS1 and to cross the BBB in the mouse (1.18% ID/g at 2 min).[58] The ^{99m}Tc complex monoamide-monoaminedithiol (MAMA) conjugated with a PIB analog (**5**) showed a high affinity on AD patient homogenates and an ability to cross the BBB (1.34% ID/g at 2 min) in the mouse despite a strong plasma protein adsorption.[59] Finally, a chalcone derivative modified with the bis-amine-bis-thiol (BAT) ^{99m}Tc complex (**6**) showed high amyloid affinity and brain penetration (1.48% ID/g at 2 min).[60] These encouraging results on BBB permeability of these SPECT probes inspired us to design MRI probes with similar chemical structures.

Fig. 2. Amyloid specific probes for SPECT imaging of amyloid aggregates.

Herein we describe a new generation of Gd-based CAs designed to detect the amyloid deposits by MRI. These probes are constituted of three parts: (i) a Gd(III)-complex as imaging moiety, (ii) an amyloid binding ligand as biovector, and (iii) a linker to bind these two entities. The Gd^{III} 3,6,9,15-tetraaza bicyclo[9.3.1]-pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (Gd(III)-PCTA) and the Gd^{III}-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (Gd(III)-DOTA) chelates were used as imaging moieties.[61] The cyclic structure of the PCTA and the DOTA ligands provides high kinetic and/or thermodynamic stability to the Gd(III) chelates necessary for *in vivo* applications.[62-63] Three biovectors, derivatives of PIB, IBOX and stilbene, were used to target the CAs on the amyloid aggregates. Different types of linkers with neutral, positive(s) or negative(s) charge(s) have been incorporated to modulate the physicochemical properties of the CAs. The affinity for the amyloid aggregates and BBB permeability of 16 CAs were then determined to establish SARs in this new class of CAs. Key structural considerations for the

future development of Gd(III)-based CAs for the detection of amyloid aggregates are highlighted by this study.

2. Chemistry.

The Gd(III)-PCTA complex modified with a carboxylic function anchored on the pyridine core (**Gd-PCTA12**) was synthesized as previously described through a pathway involving a key macrocyclisation step controlled by the template effect of Na^+ ions (Scheme 1A).[64] The Gd^{III}-1,4,7,10-tetraazacyclododecane-1-glutaric acid-4,7,10-triacetic acid (**Gd-DOTAGA**),[65] a derivative of the Gd(III)-DOTA modified with a glutaric acid linker, was synthesized by trialkylation of the cyclene in dimethylacetamide in the presence of sodium acetate (Scheme 1B). The remaining nitrogen atom was then alkylated to introduce the glutarate arm. The complex **Gd-DOTAGA** was obtained after deprotection and complexation of the ligand. Both Gd(III) chelates contain a free carboxylic acid group which was further used as an anchor for coupling a biovector or a linker.

Scheme 1. Synthesis of the imaging moieties Gd(III)-PCTA (A) and Gd(III)-DOTA (B). (i) Na_2CO_3 (4 equiv), dimethylformamide (DMF), 80 °C, 3 h (87%); (ii) 1 N NaOH, ethanol, room temperature (rt), 5 h; (iii) Et_2O -2 N HCl, dichloromethane (DCM), rt, 17 h; (iv) Gd_2O_3 (0.5 equiv), H_2O pH 4.5-5.5, 80 °C, 3 h (75% over 3 steps); (v) α -bromo-*tert*-butylacetate (3.1 equiv), AcONa (3.1 equiv), dimethylacetamide (DMA), rt, 3 weeks (60%); (vi) α -bromoethylglutarate (2.5 equiv), K_2CO_3 (2.5 equiv), acetonitrile, 80 °C, 28 h (67%); (vii) 12 N HCl, reflux, 3 d (51%); (viii) Gd_2O_3 (0.5 equiv), H_2O pH 4.5-5.5, 80 °C, 6 h (93%).

The common pathways described in the literature for the synthesis of PIB derivatives report multi-step syntheses with quite low overall yields ranging from 20 to 40% involving the Jacobson's oxidative cyclization of substituted thiobenzanilides.[51,66] We recently developed an efficient one-step synthesis of 2-arylbenzothiazole derivatives *via* a Suzuki-Miyaura cross-coupling under thermal and microwave activation.[67] The hydroxylated PIB derivative **16** was synthesized following this method from two commercially available compounds in 80% yield (Scheme 2A). Interestingly the synthon **15** can also be easily prepared in two steps from cheap reagents. A Sandmeyer-type reaction on the 2-amino-6-methoxybenzothiazole (**13**) in the presence of CuBr_2 and *tert*-butylnitrite in anhydrous conditions led to the brominated

intermediate **14** in 92% yield. The water-free conditions limited the formation of the 2-hydroxy-6-methoxybenzothiazole by-product. A careful sequential introduction of the reagents and a controlled heating time prevented the formation of the 2,7-dibromo-6-methoxybenzothiazole and 2-chloro-6-methoxybenzothiazole by-products. A deprotection of the phenolic function by BBr_3 led to the 2-bromo-6-hydroxy-benzothiazole (**15**).

Scheme 2. Synthesis of the biovectors 2-(4-*N,N*-dimethylaminophenyl)-6-hydroxybenzothiazole (**16**, A), 2-(4-*N,N*-dimethylaminophenyl)-6-hydroxybenzoxazole (**19**, B) and 4-(*N*-methylamino)-4'-hydroxy-*trans*-stilbene (**22**, C). (i) CuBr_2 (1.5 equiv), $t\text{BuONO}$ (1.5 equiv), acetonitrile, rt for 2.5 d, 65 °C for 1.5 h (92%); (ii) BBr_3 (1 M in DCM, 4.7 equiv), DCM, 0 °C to rt, 20 h (94%); (iii) $\text{Pd}(\text{dppf})\text{Cl}_2 \cdot \text{DCM}$ (5 mol%), K_2CO_3 (6.0 equiv), DMF, 80 °C, 1.5 h (80%); (iv) acyl chloride (1.1 equiv), pyridine, 0 °C to rt, 15 h; (v) K_2CO_3 (1.1 equiv), μW (300 W, 230 °C, 7-8 bars), 1 h; (vi) BBr_3 (4.7 equiv), DCM, 0 °C to rt, 15 h (20% over 3 steps); (vii) SnCl_2 (6.1 equiv), HCl (5.2 equiv), EtOH, reflux to rt, 23 h (96%); (viii) 1) paraformaldehyde (8.0 equiv), MeONa (7.0 equiv), reflux, 3 h; 2) NaBH_4 (8.0 equiv), 0 °C to reflux, 1 h (79%).

The 2-(4-*N,N*-dimethylaminophenyl)-6-hydroxybenzoxazole (**19**) was obtained in 20% yield over three steps (Scheme 2B). The condensation of the 2-fluoro-4-methoxyaniline (**17**) on the 4-dimethylaminobenzoyl chloride was followed by a cyclisation under microwave irradiation in the presence of K₂CO₃ to obtain the benzoxazole core. A final demethylation using BBr₃ led to the formation of the 2-arylbenzoxazole derivative **19**. The 4-(*N*-methylamino)-4'-hydroxy-*trans*-stilbene (**22**) was synthesized in 69% yield over 2 steps using conditions adapted from the literature (Scheme 2C).[68] The 4-nitro-4'-hydroxystilbene (**20**) was reduced with SnCl₂ and HCl in ethanol. The product was isolated by simple filtration before carrying out the mono-*N*-alkylation of the aniline with paraformaldehyde and sodium methoxide. The subsequent addition of NaBH₄ led to the monoalkylated product **22**.

Introduction of an ethylamine linker on the biovectors **16** and **19** was first attempted through a Williamson *O*-alkylation with chloroacetonitrile and K₂CO₃, followed by hydrogenation of the nitrile function in the presence of Pd/C (Scheme 3). The alkylation run smoothly but the hydrogenation step was less efficient (41% and 48% yield respectively). This was mainly caused by the generation of the hydroxylated by-products **16** and **19** resulting from the cleavage of the cyanomethyl moiety through by the probable formation of an iminium intermediate isomerised into the allyl form by interaction with Pd, as previously reported with similar reagents.[69] The reduction of the nitrile function with BH₃-THF gave the desired amine product in variable yield (25% to 68%) caused by the difficult dissociation of the final boron chelates.[70] Another approach to introduce the linker through *O*-alkylation of **16** with the *tert*-butoxycarbonyl-(*boc*)-protected chloroethylamine followed by a treatment in trifluoroacetic acid (TFA) led to compound **23** in reproducible yield (36% over 2 steps) (Scheme 3). Introduction of the

ethylamine linker on the biovector **22** was achieved using a similar pathway. Finally, peptide coupling reactions of the ethylamine terminated biovectors with the **Gd-PCTA12** or **Gd-DOTAGA** chelates led to a first library of 5 relatively small CAs (MW < 1000 Da) containing a neutral linker (Scheme 3 and Supporting Information Table S2).

Scheme 3. Synthesis of the CAs containing a neutral linker. For the synthesis of **23** and **25**: (i) *N*-(*tert*-butoxycarbonyl)-chloroethylamine (1.2 equiv), K₂CO₃ (3.3 equiv), DMF, 100 °C, 19 h (42%); (ii) DCM/TFA 4:1, rt, 1 h (85%); For the synthesis of **24**: (i) chloroacetonitrile (4.3 equiv), K₂CO₃ (1.5 equiv), acetone, 60 °C, 20 h (79%); (ii) BH₃ (11 equiv), tetrahydrofuran (THF), 70 °C, 15 h (48%); (iii) **Gd-PCTA12** or **Gd-DOTAGA** (1.0-3.1 equiv), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDCI HCl) (1.3-7.3 equiv), 1-hydroxybenzotriazole (HOBt) (0.3-0.5 equiv), H₂O/DMF or H₂O/dimethyl sulfoxide (DMSO) pH 6, rt, 1-63 h (8-65%). Eta: ethylamine linker.

The building block strategy adopted to synthesize the CAs enables easy access to a large chemical diversity for these probes. Amino acid derivatives are particularly convenient building blocks to be inserted between the Gd(III) chelates and the ethylamine terminated biovectors because of their terminal carboxylic acid and amine functions. Lysine and arginine residues were

selected because of their positively charged lateral chains under physiological conditions (*vide infra*). The coupling of the different blocks was performed through a *N*-elongation from the ethylamine terminated biovectors to the Gd(III) chelates (Scheme 4 and Supporting Information Tables S1, S2). A final treatment in TFA was performed to deprotect the boc or the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting groups of the lysine and arginine lateral chains respectively. The short deprotection time, followed by the rapid neutralisation of the Gd(III) chelate in cold water, prevented the decomplexation of the Gd³⁺ ions.

Scheme 4. Synthesis of the CAs containing a positively charged linker. (i) Fmoc(Lys-boc)OH or Fmoc(Arg-Pbf)OH (1.2-1.4 equiv), EDCI HCl (1.1-1.6 equiv), HOBt (0.2-0.4 equiv), DMF or DCM, rt, 20-72 h (79-96%); (ii) Piperidine (0.2 equiv), DMF, rt, 20 h (89%-quant); (iii) **Gd-PCTA12** or **Gd-DOTAGA** (1.0-2.6 equiv), EDCI HCl (1.3-9.2 equiv), HOBt (0.5 equiv), H₂O/DMF or H₂O/DMSO pH 6, rt, 20-62 h; (iv) TFA, rt, 1.5-2 h (8-65% over 2 steps).

The impact of multicharged linkers in the CA framework was assessed by the introduction of three charged residues on the linker. A dipeptide was conjugated on the previously modified

biovectors **31** and **33**. The *N*-9-fluorenylmethyloxycarbonyl (Fmoc) protected dilysine (**41**) and diarginine (**43**) synthons were synthesized in one coupling step by activating the carboxylic acid function of the Fmoc protected aminoacid with *N*-hydroxysuccinimide (NHS) and *N,N'*-dicyclohexylcarbodiimide (DCC) followed by the introduction of the non-protected aminoacid into the reaction mixture (Scheme 5).

Scheme 5. Synthesis of the Fmoc protected dilysine (**41**, A) and diarginine (**43**, B) synthons. (i) 1) DCC (1.1 equiv), NHS (1.3 equiv), acetonitrile, rt, 23 h; 2) H₂N(Lys-boc)OH (1.0 equiv), NaHCO₃ 6.8%/acetonitrile 1:3, 0 °C to rt, 19 h (69%); (ii) 1) DCC (1.0 equiv), NHS (1.0 equiv), DMF, rt, 17 h; 2) H₂N(Lys-boc)OH (1.0 equiv), NaHCO₃ 6.8%/DMF 1:3, 0 °C to rt, 1 h (77%).

Conjugations of the dipeptides on the modified biovectors and on the Gd(III) chelates were performed using classical peptide coupling conditions to obtain CAs carrying a trimeric positively charged linker (Scheme 6 and Supporting Information Tables S1, S2).

Scheme 6. Synthesis of the CAs containing a trimeric positively charged linker. (i) **41** or **43** (1.0 equiv), EDCI HCl (1.1-6.0 equiv), HOBt (0.2-0.5 equiv), DMF or DCM, rt, 4-20 h (37-79%); (ii) Piperidine (0.2 equiv), DMF, rt, 20 h (77%-quant); (iii) **Gd-PCTA12** or **Gd-DOTAGA** (1.5-1.7 equiv), EDCI HCl (1.3-16.7 equiv), HOBt (0.5 equiv), H₂O/DMF or H₂O/DMSO pH 6, rt, 20-45 h; (iv) TFA, rt, 1.5-2 h (8-37% over 2 steps).

Similarly, anionic linkers were introduced in the CA framework. The α -sulfo- β -alanine moiety was selected as negatively charged building block as it enables the same synthetic scheme through peptide coupling chemistry. The two Fmoc protected α -sulfo- β -alanine[71] and bis(α -sulfo- β -alaninyl)- α -sulfo- β -alanine[72] synthons were synthesized as previously reported and coupled on the ethylamine terminated biovector **31** before introducing the Gd(III) chelate **Gd-DOTAGA** (Scheme 7).

Scheme 7. Synthesis of the CAs containing a mono- or a tri-anionic linker. For the synthesis of **49**: (i) Fmoc- α -sulfo- β -alanine (1.3 equiv), EDCI HCl (2.0 equiv), HOBt (0.5 equiv), DMF, rt, 24 h (45%); (ii) Piperidine (1 equiv), DMF, rt, 15 h (96%); For the synthesis of **50**: (i) Fmoc-bis(α -sulfo- β -alaninyl)- α -sulfo- β -alanine (1.0 equiv), *O*-Benzotriazole-*N,N,N',N'*-tetramethyluronium-hexafluoro-phosphate (HBTU) (1.1 equiv), HOBt (1.1 equiv), *N,N*-diisopropylethylamine (DIEA) (3.5 equiv), DMF, rt, 2 h (79%); (ii) Piperidine (6 equiv), DMF, rt, 3 h (quant); (iii) **Gd-DOTAGA** (1.5-1.6 equiv), EDCI HCl (2.2-3.0 equiv), HOBt (0.3-0.5 equiv), H₂O or H₂O/DMF pH 6, rt, 4-24 h (41-62%).

3. Result and discussion

3.1. Affinity and physicochemical properties

The affinity of the CAs for amyloid aggregates was determined *in vitro* using [¹²⁵I]6-iodo-2-(4'-dimethylamino)-phenyl-imidazo[1,2-a]pyridine ([¹²⁵I]IMPY) binding competition experiments on synthetic A β ₁₋₄₂ aggregates (Table 1, Supporting Information Fig. S1). This radioligand has several binding sites on the amyloid aggregates and the radioligand competition assays carried out here studied the binding at the BS3 site.[73] Gd(III)-based CAs targeted on A β aggregates with a moderate *in vitro* affinity (577 nM) previously showed to be detectable *in vivo* by μ MRI

(co-injected with mannitol, T₂* weighted images).[35] CAs were considered to have an interesting affinity when $K_i < 1000$. The affinity of the CAs **28**, **29**, **37** and **52** was assessed on human Alzheimer brain homogenates. Due to their absence of affinity, the binding evaluation of these CAs on synthetic A β_{1-42} aggregates was not performed. Comparison of both methods to assess the binding affinity of the CAs was carried out with **26**, **27** and **30** and K_i in the same range were obtained (Supporting Information Table S3). The ratio $K_{i(\text{IMPY})}/K_{i(\text{CA})} \times 10^2$ was used for an accurate comparison of the CA affinities obtained from different experiments.

Table 1

Evaluation of the CA affinities on amyloid aggregates.

CA	Gd(III) chelate	Linker	Biovector	K_i (nM)	$\frac{K_i(\text{IMPY})}{K_i(\text{CA})} \times 10^2$
26	PCTA	Eta	S	217	8.3
27	PCTA	Eta	O	218	8.2
28	DOTA	Eta	S	> 1000 ^a	-
29	DOTA	Eta	O	> 1000 ^a	-
30	DOTA	Eta	Stilb	> 1000	-
34	PCTA	Lys	S	543	1.7
35	PCTA	Lys	O	> 1000	-
36	PCTA	Arg	S	152	11.8
46	PCTA	Arg ₃	S	173	5.2
37	DOTA	Lys	S	> 1000 ^a	-
38	DOTA	Lys	O	> 1000	-
39	DOTA	Arg	S	> 1000	-
51	DOTA	SO ₃	S	> 1000	-
47	DOTA	Lys ₃	S	71	25.4
48	DOTA	Arg ₃	S	131	6.9
52	DOTA	(SO ₃) ₃	S	> 1000 ^a	-
IMPY	-	-	-	5 ^a -9-18	100

^a: These CAs were evaluated on human Alzheimer brain homogenates. The biovectors are symbolized by S, O and Stilb, corresponding to the compounds **16**, **19** and **22** respectively. The linkers are symbolized by Eta, Lys, Arg and SO₃, corresponding to the ethylamine, lysine, arginine or sulfonate containing linkers respectively. The results are expressed as $K_i(\text{IMPY})/K_i(\text{CA}) \times 10^2$, the higher the ratio is, the higher the affinity is.

The first CAs assessed were Gd(III)-PCTA derivatives. This Gd(III) chelate displays interesting properties which could facilitate its detection *in vivo*. The PCTA ligand is more rigid and binds the Gd³⁺ ion *via* 7 coordination sites, which provides a theoretical interaction of two water molecules with the Gd(III) in the inner sphere of the complex while maintaining a reasonable *in vivo* stability,[74] leading to higher relaxivity compared to the Gd(III)-DOTA-based CAs

containing 8 coordination sites and consequently one water molecule in the inner sphere. An ethylamine moiety was used to link the **Gd-PCTA12** complex to the biovectors **16** and **19** to obtain the relatively small (MW 904-888 Da) and neutral CAs PCTA-Eta-BTA (**26**) and PCTA-Eta-BOX (**27**). These CAs displayed a high affinity for the amyloid aggregates with a ratio $K_{i(\text{IMPY})}/K_{i(\text{CA})} \times 10^2$ of 8.3 and 8.2 respectively (Fig. 3). However, they showed a very low solubility in water (< 1 mM) preventing their use for clinical purposes.

Fig. 3. CAs containing the neutral ethylamine linker.

Replacement of the neutral **Gd-PCTA12** complex by the anionic **Gd-DOTAGA** was expected to increase the water solubility of the CAs. This complex contains 4 acetate functions and 4 nitrogen atoms which participate in the coordination sphere of the Gd(III). The 8 sites of coordination confer to the **Gd-DOTAGA** a global anionic charge leading to high hydrosolubility.[63] As expected, the CAs DOTA-Eta-BTA (**28**) and DOTA-Eta-BOX (**29**) showed to be more soluble in water (> 3.5 mM) than the PCTA analogs **26** and **27** (Fig. 3). However, surprisingly these CAs revealed no affinity for the amyloid deposits. Changing the biovector to a different chemical family did not improve the affinity for the amyloid aggregates. Indeed, the Gd(III)-DOTA analog containing the biovector **22** (**30**, Fig. 3) displayed a high water solubility and no affinity for the amyloid aggregates.

For this reason, the investigation was pursued with Gd(III)-PCTA-based CAs. Modification of the linker was tempted to increase the water solubility of the CAs. Two Gd(III)-PCTA derivatives containing a lysine residue, PCTA-Lys-BTA (**34**) and PCTA-Lys-BOX (**35**) (Fig. 4), were synthesized and showed an increased solubility in water (1-2 mM). However, the 2-arylbenzoxazole derivative **35** did not bind the amyloid aggregates while the 2-arylbenzothiazole derivative **34** showed a moderate affinity ($K_{i(IMPY)}/K_{i(CA)} \times 10^2 = 1.7$). Replacement of the lysine residue by an arginine (PCTA-Arg-BTA, **36**, Fig. 4) led to affinity for the A β ₁₋₄₂ aggregates ($K_{i(IMPY)}/K_{i(CA)} \times 10^2 = 11.8$) and moderate solubility in water.

Fig. 4. Gd(III)-PCTA derivative CAs containing a positively charged linker.

An increased linker length to three arginine residues (PCTA-Arg₃-BTA, **46**, Fig. 4) improved slightly the water solubility of the CA while maintaining a similar affinity for the amyloid aggregates ($K_{i(\text{IMPY})}/K_{i(\text{CA})} \times 10^2 = 5.2$). In summary of our investigation on the Gd(III)-PCTA-based CAs, (i) the biovector **16** is particularly efficient for targeting CAs on amyloid aggregates, (ii) linker charge and length do not influence significantly affinity, (iii) the introduction of a mono or tri-cationic linker in the structure is required to obtain reasonably soluble CAs.

The significant water solubilising effect of the anionic Gd(III)-DOTA moiety was previously observed with CAs DOTA-Eta-BTA (**28**) and DOTA-Eta-BTX (**29**), carrying a neutral ethylamine linker. However, these two CAs showed no affinity for the amyloid aggregates.

Linker and biovector structural variations in the Gd(III)-DOTA-based CA family were then investigated. Cationic and anionic linkers of similar length were used to assess the influence of the charge on the affinity for the amyloid aggregates. For this purpose, the DOTA-Lys-BTA (**37**), the DOTA-Lys-BOX (**38**), DOTA-Arg-BTA (**39**) and the DOTA-SO₃-BTA (**51**) were synthesized (Fig. 5).

Fig. 5. Gd(III)-DOTA derivative CAs containing a monomeric charged linker.

As expected, all of these CAs were soluble in water (> 2.5-5 mM). However, none of them showed affinity for the amyloid aggregates. These results contrast with the observations made

with the Gd(III)-PCTA analogs **34** and **36** which efficiently bound the amyloid aggregates. As previously observed with the CAs **28-30**, the difference in lypophilicity, charge and/or flexibility of Gd(III)-DOTA moiety compared to Gd(III)-PCTA[62,74] seems to prevent the approach or the binding of the CA on the amyloid aggregates. The negative charge of Gd(III)-DOTA complex favours water solubility but may limit interaction with hydrophobic structures like the amyloid aggregates. On the other hand, the neutral Gd(III)-PCTA complex contains a hydrophobic pyridine moiety, which might favour interaction with these structures. These results encouraged us to synthesize Gd(III)-DOTA-based CAs with longer trimeric charged linkers to separate the Gd(III)-DOTA moiety from the biovector and limit the interaction of the Gd(III) chelate with the amyloid aggregates. For this purpose, the DOTA-Lys₃-BTA (**47**), the DOTA-Arg₃-BTA (**48**) and the DOTA-(SO₃)₃-BTA (**52**) CAs (Fig. 6) were synthesized.

Fig. 6. Gd(III)-DOTA derivative CAs containing a trimeric charged linker.

In addition to high water solubility, the CAs DOTA-Lys₃-BTA (**47**) and DOTA-Arg₃-BTA (**48**) showed high affinity for the amyloid aggregates ($K_{i(\text{IMPY})}/K_{i(\text{CA})} \times 10^2$ of 25.4 and 6.9 respectively). In contrast, the CA DOTA-(SO₃)₃-BTA (**52**) did not bind to the amyloid aggregates, highlighting the importance of linker charge on affinity. Whereas a negative charge present in the close proximity of the biovector is detrimental to the binding on amyloid aggregates, positively charged linkers are compatible with this binding as long as the anionic Gd(III)-DOTA chelate is linked to the biovector by a trimeric peptide.

3.2. Relaxivity and CMC

The longitudinal (r_1) and transversal (r_2) relaxivities of the water soluble CAs were measured under 0.47 T and 1.5 T magnetic fields at 37 °C (Table 2 and Supporting Information Tables S4, S5). These measurements were carried out in water and in reconstructed human serum (seronorm) to assess the influence of binding to blood proteins on relaxivity of the CAs. The relaxivity evaluation of some CAs was also performed in an ion cocktail to estimate the effect of ions present in blood on shielding the paramagnetic center Gd(III) from water. When the CAs did not form micelles in the concentration range assessed, a linear correlation between the longitudinal ($1/T_1$) or transversal ($1/T_2$) relaxation rates and the concentration of the CA was obtained. In contrast, when the CAs formed micelles, the plot was characterized by two straight lines of different slopes.[75] At low concentration, only the monomeric form of the CA contributed to the relaxation rate measured, whereas at higher concentration, when the CAs started to form micelles, both monomeric and micellar forms of the CA contributed to the relaxation rate, leading to higher relaxivity caused by the motion restriction of the larger micellar

structures. The straight two lines have their intersection at the critical micelle concentration (CMC) of the CA. A saturation of the relaxation value at high CA concentration was sometimes observed for measurements performed in seronorm when the binding sites on the plasma proteins were saturated by the CA.

Table 2

Measurement of longitudinal relaxivities r_1 of the CAs under 1.5 T at 37 °C.

CA	Gd(III) chelate	Linker	Biovector	r_1 water (mM ⁻¹ .s ⁻¹)		CMC in water ^a (mM)	r_1 seronorm (mM ⁻¹ .s ⁻¹)		CMC in seronorm ^a (mM)
				monomer	micelle		monomer	micelle	
28	DOTA	Eta	S	6.9	11.6	1.1	19.0	-	-
29	DOTA	Eta	O	6.5	10.7	2.0	16.1	-	-
30	DOTA	Eta	Stilb	5.5	7.8	2.6	16.1	-	-
37	DOTA	Lys	S	8.1	14.5	0.6	16.4	-	-
38	DOTA	Lys	O	7.8	11.3	1.3	14.0	-	-
39	DOTA	Arg	S	8.0	16.5	0.7	17.2	-	-
47	DOTA	Lys ₃	S	8.6	12.2	2.1	13.5	-	-
48	DOTA	Arg ₃	S	7.1	10.5	1.1	5.7	10.7	2.3
51	DOTA	SO ₃	S	8.7	-	-	nd	-	-
52	DOTA	(SO ₃) ₃	S	6.6	-	-	17.2	-	-
35	PCTA	Lys	O	12.3	-	-	21.7	-	-
36	PCTA	Arg	S	11.8	17.4	0.4	22.9	-	-
46	PCTA	Arg ₃	S	11.7 ^b	-	-	9.0	10.5	0.3

^a: CMC was determined under 1.5 T by r_1 measurements. ^b: determined under 0.47 T. The biovectors are symbolized by S, O and Stilb, corresponding to the compounds **16**, **19** and **22** respectively. The linkers are symbolized by Eta, Lys, Arg and SO₃, corresponding to the ethylamine, lysine, arginine or sulfonate containing linkers respectively.

This study revealed the ability of the Gd(III)-DOTA-based CAs to form micelles in water, characterized by CMC from 0.6 mM to 2.6 mM. This was confirmed by the profile of the plot of

relaxation against CA concentration and by the higher increase of r_2 compared to r_1 after aggregation of the CAs, a known characteristic of micellar systems (cf. Supporting Information Table S4, Fig. S2). The increase of relaxivity by self-assembling of the CAs into micelles was depending on the type of CAs. The relaxivity increase was high for the CAs **37** and **39** (79% to 185%), slightly lower for the CAs **28** and **29** (65% to 177%), moderate for the CAs **30**, **38**, **47**, **48** and **36** (40% to 90%) or low for the CA **52** (19% to 27%) (cf. Supporting Information Fig. S2). Higher relaxivity values may reflect higher water accessibility and/or lower flexibility of the Gd(III) center in the micelles. Only the two CAs carrying sulfonated linker did not self-assemble in water, which could reflect a higher solubility and/or electronic repulsion of this type of linker. The biovector also had a significant influence on CMC of the CAs (**28** vs **29** vs **30**), increasing from benzothiazole, benzoxazole to stilbene. This could be explained by an increase in solubility. Indeed, the stilbene, carrying a *N*-monomethylated aniline function, bears a hydrogen bond donor which is absent on the two other *N,N*-dimethylated biovectors, and the benzoxazole moiety is known for its higher solubility compared to the benzothiazole homolog. On the other hand, the Gd(III)-PCTA-based CAs showed a lower tendency to form micelles, potentially caused by the lipophilicity of both the PCTA ligand and the biovector, limiting the amphiphilic character of the CAs. In water, only the Gd(III)-PCTA-based CA **36** formed micelles. The lower relaxivity increase after self-assembling of this CA into micelles compared to the Gd(III)-DOTA analog CA **39** (cf. Supporting Information Fig. S2) could be explained by a decrease of water interaction with Gd(III) and/or higher flexibility of the micelle.

The r_1 of Gd(III)-DOTA derivative CAs ranged between 5.5-9.2 mM⁻¹.s⁻¹ and 7.8-18.3 mM⁻¹.s⁻¹ for the monomeric and micellar forms respectively in water, and 13.5-29.3 mM⁻¹.s⁻¹ in seronorm

except for the CA **48** (*vide infra*) (cf. Supporting Information Table S4, Fig. S3). The high relaxivity of the CAs in the seronorm is explained by a probable binding on plasma proteins.[76] Such an association is expected to reduce the CA mobility, inducing an increase in the rotational correlation time τ_R and a higher relaxivity. Plasma protein binding could also explain the non-self-assembling of most the CAs into micelles in the seronorm. The Gd(III)-PCTA homolog CAs showed slightly higher r_1 values, in the range of 11.2-12.7 mM⁻¹.s⁻¹ and 16.6-17.4 mM⁻¹.s⁻¹ for the monomeric and micellar forms respectively in water, and 21.2-33.0 mM⁻¹.s⁻¹ in the seronorm except for the CA **46** (*vide infra*) (cf. Supporting Information Table S5, Fig. S3). These differences from the Gd(III)-DOTA-based CAs can be rationalized by the different water molecule accessibility and flexibility between the two Gd(III) chelates (see above). No drop in relaxivity was observed for the measurements carried out in ionic solutions, which confirmed that endogenous ions should not interact with Gd(III) and decrease the hydration number of the CAs (cf Supporting Information Tables S4, S4). Besides, for Gd(III)-DOTA-based CAs, a shielding of the anionic charge of the chelate by ions resulted in a decrease of CMC (~1 mM).

Finally, the relaxivity of the CAs synthesized are coherent with their MW, the higher the MW of the CA, the higher τ_R and relaxivities to expect. The different linkers used do not interact significantly with the paramagnetic center since no relaxivity drop was observed for any of the compounds synthesized. These CAs are susceptible to be detected during an MRI exam if the *in vivo* Gd(III) local concentration is sufficient. This study pointed out the CAs **46** and **48** (Fig. 4 and Fig. 6) have similar relaxivity in water and in the seronorm, and they are the only CAs which

formed micelles in seronorm. This suggests a poor binding on plasma protein which could be attributed to the triarginine linker. This may play a crucial role in the biodistribution of the CAs.

3.3. BBB crossing

BBB permeability is a major challenge to overcome to obtain a suitable CA for the detection of amyloid deposits in the brain by MRI. To date, all the CAs described in the literature did not show sufficient BBB permeability to be used for *in vivo* MRI. Cationic residues were selected to both enhance water solubility and favour BBB crossing. The BBB is particularly difficult to penetrate because of tight junctions formed by epithelial cells, preventing paracellular diffusion, and because of the low presence of pinocytotic vesicles and fenestration in the membrane cells.[77] The different mechanisms known for BBB crossing can be divided into the passive and facilitated diffusion. The former is a non-energy-dependant transport driven by concentration gradient, and the latter is an active transport involving an energy-requiring step. The linkers of the CAs were designed to favour penetration of the BBB through adsorptive-mediated transcytosis. This energy-dependant and nonspecific process enables the binding and uptake of cationic residues to the negative luminal surface of endothelial cells, followed by the exocytosis at the abluminal surface. In contrary to transport across the BBB based on specific interactions with a receptor or a carrier, adsorptive-mediated transcytosis is believed to have lower restriction on the crossing substrates as far as they contain cationic charged moities.[78]

To assess the BBB permeability of the CAs, an *in vitro* model of BBB constituted of a co-culture of rat primary brain capillary endothelial cells and rat glial cells was used. This model is described as one of the most efficient to mimic the *in vivo* situation.[79-80] None of the CAs

showed efficient BBB crossing even when they were expected to be protonated at physiological pH (cf. Supporting Information Tables S6). The positive charges introduced on the CAs *via* the lysine or arginine linkers revealed not to be sufficient to induce adsorptive-mediated transcytosis across the BBB. The presence of a negative charge on the Gd(III) chelate or the relatively large size of the CAs could compete to limit the BBB crossing. The fact that no CAs could cross the BBB in spite of the large CA structural diversity assessed in this study highlights the difficulty to design a chemical structure with the right parameters to detect amyloid deposits by *in vivo* MRI.

4. Conclusion

The SARs highlighted in this paper provide key insights to design Gd-based CAs for the detection of amyloid deposits by MRI. The charge of the Gd(III)-complex is particularly important. An anionic chelate as Gd(III)-DOTA leads to water soluble CAs ($> 2.5\text{-}5\text{mM}$), but the complex needs to be separated from the biovector by a minimal peptidic chain to enable binding on amyloid aggregates. The incompatibility of amyloid aggregate binding with anionic charges was confirmed by using anionic linkers. Most of the CAs containing the Gd(III)-PCTA imaging moiety efficiently bind the amyloid aggregates. However, the water solubility of these CAs is low ($< 1\text{-}2\text{ mM}$) and the presence of a charged linker is required. A large majority of the CAs forms micelles in water but not in seronorm, suggesting an important binding on plasma proteins. Only the CAs carrying a triarginine linker show the same micelle organisation in water and in seronorm, which suggests a major role of the triarginine moiety on the interaction with plasma proteins.

Our investigation in the design of Gd-based CAs to detect amyloid aggregates did not lead to the identification of a compound with the characteristics required to make an optimal MRI probe in a clinical context. However, this study describes how structural modifications can efficiently improve hydrosolubility and amyloid aggregate affinity to obtain CAs with interesting characteristics. Based on these results, further investigations will be necessary to optimize the structures for an improved BBB crossing.

5. Experimental

5.1. Chemistry

5.1.1. General methods

All reagents were purchased from Sigma-Aldrich or Acros commercial source with the highest grade commercially available and used without additional purification. Silica gel (Geduran Si 60, 40-63 μm) and silanized silica gel (RP-2, 60, 63-200 μm) for flash column chromatography were purchased from VWR. Flash column chromatographies with RP-18 were carried out using a CombiFlash device. Thin layer chromatographies were performed using aluminium backed sheets coated with 60F254 silica gel (visualization achieved using a UV lamp at 254 nm, ninhydrin, iodine or ammonium molybdate staining). ^1H and ^{13}C NMR spectra were acquired on a Bruker AM400 400 MHz spectrometer at rt. Chemical shifts δ are given in ppm with reference to tetramethylsilane (TMS) as the internal standard. Coupling constants J are measured in Hz. Coupling patterns are described by abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), qd (quartet of doublets), m (multiplet), br (broad). Melting points were

determined using a Tottoli device. Low-resolution mass spectrometry (LRMS) analyses were performed from ionization by electrospray on positive mode, unless otherwise specified, on a Waters Micromass ZQ2000 mass spectrometer. The mass analyses were made by direct introduction carried out by infusion over 2 min of the sample dissolved in methanol-HCO₂H 0.1% (0.1 mg/mL) within a flow rate of 30 μ L/min. High-resolution mass spectrometry (HRMS) analyses were acquired on a Thermo Scientific LTQ Orbitrap mass spectrometer. The high-performance liquid chromatography (HPLC) and gas chromatography (GC) methods used are described in the Supporting Information. The water solubility of the CAs was estimated qualitatively by sight using water solutions at a concentration of 1-2 mM and 2.5-5 mM of Gd(III)-PCTA- and Gd(III)-DOTA-based CAs respectively. A lower concentration was used for the Gd(III)-PCTA derivatives because of the higher relaxivities expected for these CAs. The purity of intermediates was measured by ¹H and ¹³C NMR. The purity of the final CAs was determined by reverse-HPLC and was confirmed >95%.

5.1.2. Synthesis

5.1.2.1. *Tri-tert-butyl-1,4,7,10-tetraazacyclododecane-1,4,7-triacetate (11)*. Cyclen **10** (10.02 g, 58.14 mmol) and AcONa (14.91 g, 181.8 mmol, 3.1 equiv) were introduced in dry DMA (164 mL) under argon. α -bromo-*tert*-butylacetate (26.8 mL, 180.0 mmol, 3.1 equiv) dissolved in dry DMA (42 mL) was added dropwise at 0 °C and the reaction was performed at rt for 3 weeks. The mixture was then cooled to 0 °C and was filtrated. The precipitate was washed with cold DMA and cold EtOAc. The white solid was then solubilized in DCM and water and was extracted. The organic layer was dried over Na₂SO₄ and concentrated to obtain the compound **11** as a crystalline white powder (11.55 g, 22.47 mmol, 39%). ¹H NMR (CDCl₃, 400 MHz) δ (ppm) : 3.36 (4H, s),

3.28 (2H, s), 3.07 (4H, m), 2.88 (12H, m), 1.45-1.46 (27H, 2s). ^{13}C NMR (CDCl_3 , 100 MHz) δ (ppm) : 170.7, 169.9, 81.9, 81.7, 58.3, 51.5, 49.6, 47.7, 28.4, 28.3. LRMS (m/z): calcd for $\text{C}_{26}\text{H}_{51}\text{N}_4\text{O}_6$, 515.38 ($[\text{M}+\text{H}]^+$). Found 515.39. HPLC (method A), t_R 2.89, 3.30 min. HPLC (method C), t_R 5.81 min. Mp: 178-179 °C.

5.1.2.2. 1,4,7,10-tetraazacyclododecane-1,4,7-(tri-tert-butyl-acetate)-10-(diethyl-glutarate) (12).

The compound **11** (43.2 g, 84.0 mmol) and K_2CO_3 (29.2 g, 211 mmol, 2.5 equiv) were introduced in acetonitrile (470 mL) under argon. α -bromo-ethylglutarate (56.5 g, 211 mmol, 2.5 equiv) was introduced slowly and the reaction was performed at 80 °C for 28 h. The hot mixture was then filtrated and concentrated. 0.7 N HCl (435 mL) was added and the aqueous layer was washed with toluene (3x220 mL). After increasing the pH to 10, the aqueous layer was extracted with toluene (3x280 mL). The organic layer was washed with cold water (2x425 mL), dried over Na_2SO_4 and concentrated to obtain the compound **12** as an orange oil (39.5 g, 56.4 mmol, 67%). ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) : 4.14 (4H, m), 3.25 (7H, m), 2.80 (14 H, m), 2.50 (4H, m), 1.91 (2H, m), 1.25 (6H, m). ^{13}C NMR (CDCl_3 , 100 MHz) δ (ppm) : 173.7, 173.1, 171.2, 171.1, 80.7, 63.3, 60.3, 60.1, 56.4, 56.1, 52.7, 52.4, 52.2, 49.8, 30.8, 28.3, 25.2, 14.6, 14.3. HRMS (m/z): calcd for $\text{C}_{35}\text{H}_{64}\text{N}_4\text{NaO}_{10}$, 723.4515 ($[\text{M}+\text{Na}]^+$). Found 723.4503.

5.1.2.3. 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-10-glutaric acid. The compound **12** (35.5 g, 50.7 mmol) was dissolved in 12 N HCl (140 mL). The mixture was heated to reflux and stirred for 3 days. The mixture was then adjusted to pH 3 and concentrated to be purified by chromatography on strong basic resin. 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-10-glutaric acid (12.4 g, 26.0 mmol, 51%) was isolated as an amorphous white powder. LRMS

(*m/z*): calcd for C₁₉H₃₃N₄O₁₀, 477.2 ([M+H]⁺). Found 477.2. HPLC (method C), *t*_R 2.07 min.

HPLC (method G), *t*_R 21.42 min.

5.1.2.4. Gadolinium 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-10-glutaric acid (Gd-DOTAGA). 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-10-glutaric acid (12.2 g, 20.5 mmol) and Gd₂O₃ (3.73 g, 10.3 mmol, 0.5 equiv) were dissolved in H₂O (100 mL). The mixture was heated to 80 °C for 6 h maintaining the pH to 4.5-5.5. The mixture was then filtrated and concentrated. The crude was purified on silanized silica gel RP-2 (H₂O/MeOH gradient) and on Chelex resin. The aqueous layer was concentrated by lyophilization and the compound **Gd-DOTAGA** (15.1 g, 24.0 mmol, 93%) was isolated as a white slightly electrostatic powder. HRMS (*m/z*): calcd for C₁₉H₂₈GdN₄Na₂O₁₀, 676.0836 ([M-H+2Na]⁺). Found 676.0838. HPLC (method A), *t*_R 4.09 min.

5.1.2.5. 2-bromo-6-methoxybenzothiazole (14). Cupper (II) bromide (18.28 g, 81.01 mmol, 1.5 equiv) and *tert*-butyl nitrite (10.9 mL, 81.8 mmol, 1.5 equiv) were dissolved in anhydrous acetonitrile under argon. 2-amino-6-methoxybenzothiazole (**13**) (9.80 g, 53.3 mmol) was introduced and the mixture was stirred at rt for 2.5 days, before heating to 65 °C for 1.5 h. The mixture was then carefully poured in a solution of 3 N HCl and was extracted with Et₂O (3x250 mL). The organic layer was washed with 3 N HCl (400 mL) and brine (600 mL) before drying over anhydrous MgSO₄ and concentration. The crude was triturated in MeOH and filtrated. The filtrate was concentrated and purified by flash chromatography on silica gel (cyclohexane/EtOAc 97.5:2.5). The compound **14** (12.01 g, 49.22 mmol, 92%) was isolated as a white amorphous powder. ¹H NMR (DMSO-*d*₆, 400 MHz) δ(ppm) : 7.87 (1H, d, *J* = 9.0 Hz), 7.69 (1H, d, *J* = 2.5 Hz), 7.12 (1H, dd, *J* = 2.5, 9.0 Hz), 3.82 (3H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ(ppm) : 157.1, 146.3, 138.2, 135.9,

122.8, 116.0, 104.8, 55.8. LRMS (m/z): calcd for C_8H_7BrNOS , 243.94-245.94 ($[M+H]^+$). Found 243.93-245.93. HPLC (method A), t_R 5.41 min. HPLC (method G), t_R 37.55 min. HPLC (method H), t_R 4.73 min. HPLC (method I), t_R 6.62 min. GC (method L), t_R 6.72 min. Mp: 92 °C.

5.1.2.6.1. 2-bromo-6-hydroxybenzothiazole (15). The compound **14** (5.369 g, 22.00 mmol) was dissolved in anhydrous DCM (105 mL) under argon. The mixture was cooled to 0 °C and BBr_3 (1 M in DCM, 103 mL, 103 mmol, 4.7 equiv) was added dropwise. The mixture was stirred at 0 °C for 10 min and at rt for 27 h. The reaction was stopped by carefully pouring the mixture in MeOH at 0 °C before concentration. The crude oil was mixed in a solution of DCM/EtOAc/MeOH 10:10:1 (15 mL) and was filtrated to isolate the precipitate subsequently washed with the solution of DCM/EtOAc/MeOH 10:10:1 (3x25 mL). The compound **15** (5.686 g, 24.71 mmol, 94%, molar purity 87% by 1H NMR) was obtained as a beige amorphous powder and used for the next step without further treatment. The product contained a minor amount of the by-product 2-bromo-6-hydroxy-7-bromobenzothiazole (13% molar by 1H NMR). 1H NMR ($DMSO-d_6$, 400 MHz) δ (ppm) : 9.98 (1H, s), 7.78 (1H, d, J = 8.8 Hz), 7.40 (1H, d, J = 2.5 Hz), 6.97 (1H, dd, J = 2.5, 8.8 Hz). ^{13}C NMR ($DMSO-d_6$, 100 MHz) δ (ppm) : 156.0, 145.3, 138.2, 134.6, 122.9, 116.2, 106.7. LRMS (m/z): calcd for C_7H_5BrNOS , 229.93-231.93 ($[M+H]^+$). Found 229.93-231.93. HPLC (method A), t_R 4.17 min. HPLC (method G), t_R 29.68 min. HPLC (method H), t_R 5.51 min. GC (method L), t_R 7.35 min. GC (method M), t_R 3.00 min.

5.1.2.6.2. By-product 2-bromo-6-hydroxy-7-bromobenzothiazole. 1H NMR ($DMSO-d_6$, 400 MHz) δ (ppm) : 10.90 (1H, s), 7.83 (1H, d, J = 8.8 Hz), 7.17 (1H, d, J = 8.8 Hz). ^{13}C NMR ($DMSO-d_6$, 100 MHz) δ (ppm) : 153.2, 144.5, 140.8, 134.2, 122.3, 116.4, 98.3. HPLC (method G), t_R 13.73 min. GC (method L), t_R 7.64 min.

5.1.2.7. 2-(4-*N,N*-dimethylaminophenyl)-6-hydroxybenzothiazole (**16**). The compound **15** (5.194 g, 22.58 mmol) and 4-aminophenylboronic acid (4.620 g, 28.00 mmol, 1.2 equiv) were dissolved in a mixture of DMF/K₂CO₃ 2 M 65:35 (188 mL) (K₂CO₃, 7.8 mmol, 6.0 equiv). After 30 min under argon bubbling, Pd(dppf)Cl₂.DCM (816.6 mg, 1.116 mmol, 0.05 equiv) was introduced and the reaction was performed at 80 °C for 1.5 h. The mixture was then filtered on Celite® and concentrated. The crude was used without further purification for the next step. The product could also be isolated by a precipitation process. In this case, the crude was dissolved in MeOH-1 N HCl and Et₂O was introduced to induce the formation of the precipitate isolated by filtration. The precipitate was then poured into water (20 mL) and pH was adjusted to 6. The compound **16** (2.782 g, 10.47 mmol, 80%) was isolated by filtration as a brown amorphous powder. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 9.72 (1H, s), 7.81 (1H, d, *J* = 8.9 Hz), 7.73 (2H, d, *J* = 8.7 Hz), 7.33 (1H, d, *J* = 2.4 Hz), 6.92 (1H, dd, *J* = 2.4, 8.7 Hz), 6.81 ppm (2H, d, *J* = 9.0 Hz), 3.01 (6H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 164.2, 155.0, 151.8, 147.4, 135.2, 127.9, 122.5, 120.6, 115.5, 111.9, 106.7, 39.5. HRMS (*m/z*): calcd for C₁₅H₁₅N₂OS, 271.0900 ([M+H]⁺). Found 271.0899. HPLC (method A), *t*_R 5.18 min. HPLC (method G), *t*_R 33.20 min. HPLC (method H), *t*_R 7.28 min. HPLC (method J), *t*_R 25.07 min. GC (method M), *t*_R 8.00 min.

5.1.2.8. 2-(4-*N,N*-dimethylaminophenyl)-6-cyanomethoxybenzothiazole. The compound **16** and K₂CO₃ (4.773 g, 34.58 mmol, 1.5 equiv) were introduced in acetone (305 mL). Chloroacetonitrile (6.61 mL, 102 mmol, 4.5 equiv) was poured in the mixture which was stirred at reflux for 6 h. The hot mixture was filtrated on Celite® and the filtrate was kept 3 h at rt to favour the formation of a precipitate isolated by filtration and washed with Et₂O. 2-(4-*N,N*-dimethylaminophenyl)-6-cyanomethoxybenzothiazole (5.189 g, 16.77 mmol, 74% over 2 steps) was obtained as a white amorphous powder. The filtrate could be concentrated and purified by

chromatography on silica gel (DCM). The product-containing fraction isolated was taken up and mixed into Et₂O before filtration to get an added amount of 2-(4-*N,N*-dimethylaminophenyl)-6-cyanomethoxybenzothiazole. This procedure enhanced the yield to 80-91%. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 7.90 (1H, d, *J* = 8.9 Hz), 7.85 (2H, d, *J* = 8.8 Hz), 7.80 (1H, d, *J* = 2.5 Hz), 7.20 (1H, dd, *J* = 2.5, 8.9 Hz), 6.82 (2H, d, *J* = 8.8 Hz), 5.25 (2H, s), 3.02 (6H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 166.7, 153.7, 152.1, 149.6, 135.1, 128.3, 122.7, 120.1, 116.6, 115.5, 111.8, 107.0, 54.2, 39.7. HRMS (*m/z*): calcd for C₁₇H₁₆N₃OS, 310.1009 ([M+H]⁺). Found 310.1001. HPLC (method A), *t*_R 6.38 min. HPLC (method G), *t*_R 37.87 min. HPLC (method H), *t*_R 7.40 min. GC (method M), *t*_R 8.80 min.

5.1.2.9. 2-(4-N,N-dimethylaminophenyl)-6-cyanomethoxybenzoxazole. 2-(4-*N,N*-dimethylaminophenyl)-6-hydroxybenzoxazole (**19**) (1.809 g, 7.120 mmol) and K₂CO₃ (1.091 g, 7.895 mmol, 1.1 equiv) were introduced in acetone (27 mL). Chloroacetonitrile (1.49 mL, 23.1 mmol, 3.3 equiv) was added and the mixture was stirred at reflux. After 15h, a supplement amount of K₂CO₃ (367 mg, 2.66 mmol, 0.37 equiv) and chloroacetonitrile (0.46 mL, 7.1 mmol, 1.0 equiv) was added and the mixture was stirred at reflux for another 5 h. The hot mixture was filtrated on Celite® and the filtrate was kept 1 h at rt to favour the formation of a precipitate filtrated and washed with Et₂O. A white amorphous powder (1.109 g, 3.781 mmol, 53%) was isolated. The filtrate could be concentrated and purified by chromatography on silica gel (DCM). The product-containing fraction isolated was taken up and mixed into Et₂O before filtration to get an added amount of 2-(4-*N,N*-dimethylaminophenyl)-6-cyanomethoxybenzoxazole (547.0 mg, 1.865 mmol). This procedure enhanced the yield to 79%. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 7.97 (2H, d, *J* = 9.0 Hz), 7.67 (1H, d, *J* = 8.7 Hz), 7.53 (1H, d, *J* = 2.5 Hz), 7.07 (1H, dd, *J* = 2.5, 8.7 Hz), 6.86 (2H, d, *J* = 9.0 Hz), 5.26 (2H, s), 3.03 (6H, s). ¹³C NMR (DMSO-*d*₆, 100

MHz) δ (ppm) : 163.7, 154.3, 152.6, 150.9, 137.6, 128.7, 120.0, 117.0, 113.2, 112.1, 97.9, 54.8, 40.0. HRMS (m/z): calcd for $C_{17}H_{16}N_3O_2$, 294.1237 ($[M+H]^+$). Found 294.1237. HPLC (method C), t_R 8.76 min. HPLC (method G), t_R 36.23 min. GC (method L), t_R 12.4 min.

*5.1.2.10.1. 2-(4-*N,N*-dimethylaminophenyl)-6-aminoethoxybenzothiazole (23). Method 1.* 2-(4-*N,N*-dimethylaminophenyl)-6-cyanomethoxybenzothiazole (3.353 g, 10.85 mmol) was dissolved in anhydrous THF (250 mL). BH_3 -THF (1 M in DCM, 123.9 mL, 123.9 mmol, 11 equiv) was added at rt and the mixture was stirred at 70 °C for 15 h. The reaction mixture was then poured carefully into MeOH and stirred for 3 h. After evaporation, the crude was mixed in water and the pH was increased to 13 (2 N NaOH). The aqueous layer was extracted with DCM, dried over Na_2SO_4 , concentrated and purified by flash chromatography on silica gel ($CHCl_3/MeOH$ 95:5 to 9:1). The compound **23** (1.802 g, 5.749 mmol, 54%) was isolated as a yellow amorphous powder. 1H NMR ($DMSO-d_6$, 400 MHz) δ (ppm) : 7.82 (1H, d, J = 8.9 Hz), 7.81 (1H, d, J = 8.9 Hz), 7.62 (1H, d, J = 2.5 Hz), 7.07 (1H, dd, J = 2.5, 8.9 Hz), 6.81 (2H, d, J = 8.9 Hz), 4.01 (2H, t, J = 5.7 Hz), 3.01 (6H, s), 2.93 (2H, t, J = 5.7 Hz). ^{13}C NMR ($DMSO-d_6$, 100 MHz) δ (ppm) : 165.4, 156.1, 151.9, 148.3, 135.2, 128.1, 122.4, 120.5, 115.6, 111.8, 105.7, 70.2, 40.7, 39.7. HRMS (m/z): calcd for $C_{17}H_{20}N_3OS$, 314.1322 ($[M+H]^+$). Found 314.1317. HPLC (method A), t_R 3.12 min. HPLC (method G), t_R 23.64 min. HPLC (method J), t_R 21.83 min. GC (method M), t_R 9.1 min.

*5.1.2.10.2. 2-(4-*N,N*-dimethylaminophenyl)-6-aminoethoxybenzothiazole (23). Method 2.* 2-(4-*N,N*-dimethylaminophenyl)-6-(*N*-(*tert*-butoxycarbonyl)-ethoxyamine)-benzothiazole (119 mg, 0.285 mmol) was dissolved in DCM/TFA 4:1 (2 mL) and stirred at rt for 1 h. Then the

mixture was concentrated and mixed in Et₂O before filtration. The compound **23** (104 mg, 0.243 mmol, 85%) was isolated as an orange electrostatic powder (triflate salt).

5.1.2.11. 2-(4-N,N-dimethylaminophenyl)-6-aminoethoxybenzoxazole (24). 2-(4-N,N-dimethylaminophenyl)-6-cyanomethoxybenzothiazole (1.500 g, 5.119 mmol) was dissolved in anhydrous THF (17 mL). BH₃-THF (1 M in DCM, 56.3 mL, 56.3 mmol, 11 equiv) was added at rt and the mixture was stirred at 70 °C for 2 h. The reaction mixture was then poured carefully into MeOH and stirred for 15 min. After evaporation, the crude was dissolved in EtOAc/2.5 N HCl 2:1 (120 mL) and mixed at rt for 15 min. The mixture was then cooled to 0 °C and the pH was increased to 12 (2 N NaOH). The aqueous layer was extracted with EtOAc (3x60 mL), dried over MgSO₄, concentrated and purified by flash chromatography on silica gel (DCM/MeOH 98:2 to 9:1). The compound **24** (730.0 mg, 2.458 mmol, 48%) was isolated as a black oil. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 7.93 (2H, d, *J* = 9.0 Hz), 7.55 (1H, d, *J* = 8.5 Hz), 7.32 (1H, d, *J* = 2.5 Hz), 6.94 (1H, dd, *J* = 2.5, 8.5 Hz), 6.83 (2H, d, *J* = 9.0 Hz), 3.98 (2H, t, *J* = 5.7 Hz), 3.01 (6H, s), 2.91 (2H, t, *J* = 5.7 Hz). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 162.5, 156.6, 152.1, 150.8, 135.6, 128.1, 118.8, 113.3, 112.7, 111.8, 96.4, 70.9, 39.7. HPLC (method C), *t*_R 2.98 min. HPLC (method G), *t*_R 36.6 min. GC (method L), *t*_R 12.43 min. HRMS (*m/z*): calcd for C₁₇H₂₀N₃O₂, 298.1550 ([M+H]⁺). Found 298.1545.

5.1.2.12. N-(tert-butyloxycarbonyl)-chloroethylamine. Chloroethylamine chlorhydrate (2.500 g, 2.134 mmol), bis-*tert*-butyloxycarbonyl anhydride (5.589 g, 2.561 mmol, 1.2 equiv) and Et₃N (6.57 mL, 4.80 mmol, 2.2 equiv) were introduced in acetonitrile (20 mL). The mixture was vigorously stirred at rt for 24 h. Then, the mixture was filtrated on Celite® and concentrated. The crude was mixed with water and extracted with DCM (3x70 mL). The organic layer was

dried over MgSO_4 and concentrated. *N*-(*tert*-butyloxycarbonic acid)-chloroethylamine (3.825 g, 2.129 mmol, quantitative) was isolated as a translucent oil. ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ (ppm) : 7.07 (1H, s), 3.55 (2H, t, $J = 6.4$ Hz), 3.22 (2H, q, $J = 6.4$ Hz), 1.38 (9H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz) δ (ppm) : 155.5, 78.0, 43.4, 42.0, 28.2.

5.1.2.13. 2-(4-*N,N*-dimethylaminophenyl)-6-(*N*-(*tert*-butyloxycarbonic acid)-ethoxyamine)-benzothiazole. The compound **16** (385.2 g, 1.427 mmol), *N*-(*tert*-butyloxycarbonic acid)-chloroethylamine (309.6 mg, 1.723 mmol, 1.2 equiv) and K_2CO_3 (659 mg, 4.78 mmol, 3.3 equiv) were introduced in DMF (8 mL). The mixture was stirred at 100 °C for 19 h. Then the mixture was concentrated and purified by flash chromatography on silica gel (cyclohexane/DCM from 1:1 to 0:1). 2-(4-*N,N*-dimethylaminophenyl)-6-(*N*-(*tert*-butyloxycarbonic acid)-ethoxyamine)-benzothiazole (248 mg, 0.600 mmol, 42%) was isolated as a yellow amorphous powder. ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ (ppm) : 7.83 (2H, d, $J = 8.9$ Hz), 7.80 (1H, d, $J = 8.9$ Hz), 7.62 (1H, d, $J = 2.5$ Hz), 7.05 (2H, dd, $J = 2.5, 8.9$ Hz), 6.80 (2H, d, $J = 8.9$ Hz), 4.03 (2H, t, $J = 5.8$ Hz), 3.33 (2H, t, $J = 5.8$ Hz), 3.01 (6H, s), 1.39 (9H, s). ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz) δ (ppm) : 165.5, 155.9, 151.9, 148.4, 135.2, 128.1, 122.4, 120.5, 115.6, 111.9, 105.7, 77.8, 67.1, 39.5, 28.2. HRMS (m/z): calcd for $\text{C}_{22}\text{H}_{28}\text{N}_3\text{O}_3\text{S}$, 414.1846 ($[\text{M}+\text{H}]^+$). Found 414.1844. HPLC (method A), t_R 7.24 min. HPLC (method J), t_R 34.63 min.

5.1.2.14. 4-amino-4'-hydroxy-*trans*-stilbene (21). 4-nitro-4'-hydroxy-*trans*-stilbene (**20**) (9.850 g, 40.83 mmol) was introduced in EtOH (300 mL). SnCl_2 (47.25 g, 249.2 mmol, 6.1 equiv) and 12 N HCl (17.7 mL, 212 mmol, 5.2 equiv) were added and the mixture was stirred at reflux for 3 h, before decreasing the temperature to rt keeping stirring for 20 h. Then, the mixture was filtrated and the precipitate was washed with Et_2O . The compound **21** (8.310 g, 37.56 mmol) was

isolated as a red amorphous powder. ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm) : 7.61 (2H, d, J = 8.6 Hz), 7.42 (2H, d, J = 8.7 Hz), 7.29 (2H, d, J = 8.6 Hz), 7.15 (1H, d, J = 16.4 Hz), 7.02 (1H, d, J = 16.4 Hz), 6.78 (2H, d, J = 8.7 Hz). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 157.5, 136.5, 129.1, 128.0, 127.9, 127.1, 123.9, 122.7, 115.6. LRMS (m/z): calcd for $\text{C}_{14}\text{H}_{14}\text{NO}$, 212.11 ($[\text{M}+\text{H}]^+$). Found 212.09. HPLC (method A), t_{R} 2.52 min. HPLC (method G), t_{R} 19.8-20.8 min.

5.1.2.15. 4-(*N*-methylamino)-4'-hydroxy-trans-stilbene (22). The compound **21** (4.80 g, 22.7 mmol) and paraformaldehyde (4.83, 167 mmol, 8.0 equiv) were added to a freshly prepared solution of MeONa in MeOH under argon (3.66 g, 159 mmol, 7.0 equiv of Na (s) in 65 mL of MeOH). The mixture was stirred at reflux for 3 h before being cooled to 0 °C. Then NaBH_4 (6.883 g, 182.0 mmol, 8.0 equiv) was introduced and the mixture was stirred at reflux for 1 h. Then, MeOH was evaporated and water was introduced. After adjusting the pH to 6, the mixture was filtrated and the precipitate was washed with Et_2O , taken up in MeOH, and dried over MgSO_4 . The compound **22** (3.690 g, 16.38 mmol, 72%) was isolated as a grey amorphous powder. ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm) : 9.38 (1H, s), 7.31 (2H, d, J = 8.6 Hz), 7.28 (2H, d, J = 8.7 Hz), 6.85 (1H, d, J = 16.4 Hz), 6.79 (1H, d, J = 16.4 Hz), 6.72 (2H, d, J = 8.6 Hz), 6.51 (2H, d, J = 8.7 Hz), 5.77 (1H, br), 2.68 (3H, s). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 156.4, 149.2, 129.0, 127.1, 127.0, 125.9, 125.1, 123.0, 115.4, 111.7, 29.6. HRMS (m/z): calcd for $\text{C}_{15}\text{H}_{16}\text{NO}$, 226.1226 ($[\text{M}+\text{H}]^+$). Found 226.1223. HPLC (method A), t_{R} 3.54 min. HPLC (method G), t_{R} 22.97 min. GC (method N), t_{R} 6.97 min.

5.1.2.16.1. 4-(*N*-methylamino)-4'-ethoxyamine-trans-stilbene (25). The compound **22** (1.505 g, 6.680 mmol), *N*-(*tert*-butoxycarbonyl)-chloroethylamine (1.442 g, 8.027 mmol, 1.2 equiv) and K_2CO_3 (3.087 g, 22.34 mmol, 3.3 equiv) were introduced in DMF (8 mL). The mixture was

stirred at 100 °C for 42 h. Then the mixture was concentrated and purified by flash chromatography on silica gel (DCM/MeOH from 1:0 to 9:1). 4-(*N*-methylamino)-4'-*N*-(*tert*-butyloxycarbonyl)-ethoxyamine-*trans*-stilbene (567.1 mg, 1.539 mmol, 23%) was isolated as a yellow amorphous powder. The product was then introduced in DCM/TFA 4:1 (12 mL) and stirred for 1 h at rt. The crude was mixed with water pH 10 and extracted with DCM. The organic layer was dried over MgSO₄, concentrated and purified by flash chromatography on silica gel (DCM/MeOH 9:1). The compound **25** (412.5 mg, 1.537 mmol, quant) was isolated as a yellow amorphous powder. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 8.10 (1H, br), 7.47 (2H, d, *J* = 8.7 Hz), 7.34 (2H, d, *J* = 8.6 Hz), 6.95 (4H, m), 6.60 (2H, d, *J* = 8.5 Hz), 4.17 (2H, t, *J* = 5.0 Hz), 3.22 (2H, m), 2.71 (3H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 156.8, 148.5, 131.3, 127.4, 127.1, 127.1, 122.9, 118.2, 114.9, 112.6, 64.4, 38.4, 30.2. HRMS (*m/z*): calcd for C₁₇H₂₁N₂O, 269.1648 ([M+H]⁺). Found 269.1647. HPLC (method A), *t*_R 1.12 min. HPLC (method G), *t*_R 15.90 min.

5.1.2.16.2. *Protected intermediate*. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 7.42 (2H, d, *J* = 8.8 Hz), 7.30 (2H, d, *J* = 8.7 Hz), 7.1-6.4 (4H, m), 6.52 (2H, d, *J* = 8.7 Hz), 3.94 (2H, m), 3.28 (2H, m), 2.68 (3H, s), 1.39 (9H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 157.3, 155.7, 149.4, 130.8, 127.3, 126.9, 125.4, 124.9, 122.5, 114.7, 111.7, 77.8, 66.4, 38.2, 29.6, 28.2. HRMS (*m/z*): calcd for C₂₂H₂₉N₂O₃, 369.2173 ([M+H]⁺). Found 369.2174. HPLC (method A), *t*_R 6.04 min. HPLC (method G), *t*_R 31.81 min.

5.1.2.17. *N_α-Fmoc-bis-[N_ε-(tert-butyloxycarbonyl)-L-lysine] (Fmoc-Lys(boc)-Lys(boc)-OH) (41)*. *N_α-Fmoc-N_ε-(tert-butyloxycarbonyl)-L-lysine (40)* (500 mg, 1.05 mmol) and NHS (159 mg, 1.36 mmol, 1.3 equiv) were introduced in anhydrous acetonitrile (3 mL) under argon. Then DCC (242

mg, 1.16 mmol, 1.1 equiv) was added and the mixture was stirred at rt for 23 h (preparation **A**). In a separate round-bottom flask, (*N*_ε-*tert*-butyloxycarbonyl)-L-lysine (263 mg, 1.07 mmol, 1.0 equiv) was stirred in a mixture of NaHCO₃ 6.8%/acetonitrile 1:2 (9 mL) at 0 °C for 10 min (preparation **B**). The preparation **B** was then poured dropwise into the preparation **A** at 0 °C. The mixture was stirred for 19 h at rt before adjusting the pH to 6 (1 N HCl). Then, acetonitrile was evaporated and the aqueous layer was acidified to pH 1 (12 N HCl) before extraction with DCM.(3x10 mL). The organic layer was washed with water (1x30 mL) and dried over MgSO₄. The crude was purified by flash chromatography on silica gel (DCM/MeOH from 1:0 to 95:5). The compound **41** (560 mg, 0.721 mmol, 69%) was obtained as a white amorphous powder. ¹H NMR (CD₃OD, 400 MHz) δ(ppm) : 7.78 (2H, d, *J* = 7.4 Hz), 7.66 (2H, t, *J* = 7.4 Hz), 7.38 (2H, t, *J* = 7.4 Hz), 7.30 (2H, t, *J* = 7.4 Hz), 4.36 (2H, dd, *J* = 4.4-6.8 Hz), 4.28 (1H, m), 4.21 (1H, t, *J* = 6.8 Hz), 4.11 (1H, m), 3.01 (4H, m), 1.84-1.67 (4H, 2m), 1.55-1.30 (8H, m), 1.42-1.38 (18H, 2s). ¹³C NMR (CD₃OD, 100 MHz) δ(ppm) : 174.5, 158.6, 145.4, 145.1, 142.6, 128.8, 128.2, 126.3, 120.9, 79.9, 79.8, 68.0, 56.5, 48.4, 41.2, 41.1, 32.9, 32.8, 31.6, 31.5, 28.81, 28.79, 24.2, 24.1. HRMS (*m/z*): calcd for C₃₇H₅₂N₄NaO₉, 719.3627 ([M+Na]⁺). Found 719.3625. HPLC (method A), *t*_R 7.02 min. HPLC (method C), *t*_R 10.24 min. HPLC (method G), *t*_R 39.48 min (activated starting material, *t*_R 39.10 min).

5.1.2.18. *N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine (*H*₂*N*-Arg(*Pbf*)-*OH*). *N*_α-Fmoc-*N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine (4.520 g, 6.827 mmol) and piperidine (810 μL, 8.11 mmol, 1.2 equiv) were introduced in DMF (90 mL). The mixture was stirred at rt for 15 h before being concentrated, stirred in Et₂O (200 mL) for 30 min and then filtrated. *N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine (2.683 g, 6.292 mmol, 92%) was isolated as a white amorphous powder. ¹H NMR (CD₃OD, 400

MHz) δ (ppm) : 7.90 (1H, s), 3.53 (1H, t, J = 6.4 Hz), 3.35 (1H, s), 3.20 (2H, m), 3.00 (2H, s), 2.57 (3H, s), 2.51 (3H, s), 2.08 (3H, s), 1.83 (2H, m), 1.63 (2H, m), 1.45 (6H, s). ^{13}C NMR (CD_3OD , 100 MHz) δ (ppm) : 159.9, 158.2, 154.9, 139.4, 134.3, 133.5, 126.0, 118.5, 87.7, 55.7, 44.0, 29.6, 28.7, 26.4, 19.5, 18.3, 15.4, 12.5. LRMS (m/z): calcd for $\text{C}_{19}\text{H}_{31}\text{N}_4\text{O}_4\text{S}$, 427.25 ($[\text{M}+\text{H}]^+$). Found 427.40. HPLC (method D), t_{R} 11.10 min.

5.1.2.19. *N $_{\alpha}$ -Fmoc-bis-[N $_{\omega}$ -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine] (Fmoc-Arg(Pbf)-Arg(Pbf)-OH) (43).* *N $_{\alpha}$ -Fmoc-N $_{\omega}$ -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine* **42** (4.000 g, 6.042 mmol) and NHS (0.717 g, 6.11 mmol, 1.0 equiv) were introduced in anhydrous DMF (60 mL) under argon. Then DCC (1.274 g, 6.113 mmol, 1.0 equiv) was added to the mixture which was stirred at rt for 17 h (preparation **A**). In a separate round-bottom flask, *N $_{\omega}$ -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine* (2.574 g, 6.045 mmol, 1.0 equiv) was stirred in a mixture of NaHCO_3 6,8%/DMF 2:3 (100 mL) at 0 °C for 10 min (preparation **B**). The preparation **A** was then poured dropwise into the preparation **B** at 0 °C. The mixture was stirred for 1 h at rt. After acidification to pH 1 (12 N HCl), the mixture was extracted with DCM (3x100 mL). The organic layer was washed with water (1x200 mL) and dried over MgSO_4 . The compound **43** (4.944 g, 4.677 mmol, 77%) was isolated by flash chromatography on silica gel (DCM/MeOH from 1:0 to 9:1) as a white amorphous powder. ^1H NMR (CD_3OD , 400 MHz) δ (ppm) : 7.77 (2H, d, J = 7.4 Hz), 7.64 (2H, t, J = 7.4 Hz), 7.36 (2H, t, J = 7.4 Hz), 7.28 (2H, t, J = 7.4 Hz), 4.36 (3H, m), 4.18 (1H, m), 4.11 (1H, m), 3.15 (4H, m), 2.96 (2H, s), 2.95 (2H, s), 2.57 (3H, s), 2.55 (3H, s), 2.51 (3H, s), 2.49 (3H, s), 2.06 (3H, s), 2.05 (3H, s, H19), 1.87 (2H, m), 1.78 (2H, m), 1.65 (2H, m), 1.55 (2H, m), 1.41 (3H, s), 1.42 (3H, s). ^{13}C NMR (CD_3OD , 100 MHz) δ (ppm) : 175.3, 170.7, 159.9, 159.8, 158.4, 158.1, 145.4, 145.1, 142.6, 139.42, 139.39, 134.3, 133.5, 128.8, 128.2, 126.2, 126.0, 120.9, 118.5, 87.7, 67.9, 56.0,

48.4, 43.9, 30.8, 30.1, 30.4, 30.3, 28.7, 19.60, 19.58, 18.40, 18.38, 12.5. HRMS (m/z): calcd for $C_{53}H_{69}N_8O_{11}S_2$, 1057.4522 ($[M+H]^+$). Found 1057.4517. HPLC (method A), t_R 7.72 min. HPLC (method D), t_R 4.08 min. HPLC (method G), t_R 42.41 min.

5.1.2.20. *α -Sulfo- β -alanine*. Oleum 30% (147 mL) was added to β -alanine (9.990 g, 112.2 mmol) in a round-bottom flask at 0 °C. The reaction was carried out for 6 days at rt. Then the mixture was cooled to 0 °C and H₂O (300 mL) was cautiously added. The mixture was kept at 0 °C during 4 days before filtration. The precipitate was dried over P₂O₅. *α -Sulfo- β -alanine* (11.33 g, 67.04 mmol, 60%) was isolated as a white amorphous powder. ¹H NMR (D₂O, 400 MHz) δ (ppm) : 4.27 (1H, t, J = 6.2 Hz), 3.67 (2H, m). ¹³C NMR (D₂O, 100 MHz) δ (ppm) : 168.7, 61.7, 37.5. LRMS (negative mode) (m/z): calcd for $C_3H_6NO_5S$, 168.00 ($[M-H]^-$). Found 168.01.) HPLC (method E), t_R 2.06 min.

5.1.2.21. *N-(9-Fluorenylmethyloxycarbonyl)- α -sulfo- β -alanine*. *α -Sulfo- β -alanine* (501 mg, 2.96 mmol) was dissolved in a solution of Na₂CO₃ 8.5% (7 mL) at 0 °C. A solution of 9-fluorenylmethyl *N*-succinimidyl carbonate (819 mg, 2.38 mmol, 0.8 equiv) in 1,4-dioxane (5 mL) was introduced dropwise and the mixture was stirred for 22 h at rt. Then, the pH was adjusted to 2 (12 N HCl) before concentration and the crude was dissolved in hot water (50 °C) to be purified by chromatography on silanized silica gel RP-2 (H₂O/MeOH from 1:0 to 9:1). *N-(9-Fluorenylmethyloxycarbonyl)- α -sulfo- β -alanine* (829.1 mg, 2.120 mmol, 71%) was isolated as a white amorphous powder. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 7.88 (2H, d, J = 7.3 Hz), 7.68 (2H, m), 7.41 (2H, t, J = 7.3 Hz), 7.32 (2H, t, J = 7.3 Hz), 4.22 (2H, m), 3.62 (1H, m), 3.47 (1H, m). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 169.5, 156.0, 143.9, 140.7, 127.7, 127.2, 125.4, 120.2, 65.6, 65.0, 46.7, 40.3. LRMS (negative mode) (m/z): calcd for $C_{18}H_{16}NO_7S$,

390.06 ($[M-H]^-$). Found 390.16. HPLC (method A), t_R 5.02 min. HPLC (method E), t_R 24.20 min. HPLC (method J), t_R 24.09 min.

5.1.2.22. *N*-(9-Fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alanine. *N*-(9-Fluorenylmethyloxycarbonyl)- α -sulfo- β -alanine (1.000 g, 2.558 mmol), NHS (421.7 mg, 3.591 mmol, 1.4 equiv) and DCC (533.3 mg, 2.559 mmol, 1.0 equiv) were introduced in anhydrous DMF (20 mL) under argon. The mixture was stirred at rt for 19 h and was then poured dropwise into a stirred solution of $NaHCO_3$ 6.8% (11.5 mL) at 0 °C containing α -sulfo- β -alanine (429.9 mg, 2.544 mmol, 1.0 equiv). The mixture was stirred at rt for 3 h before adjusting the pH to 2 (12 N HCl). After concentration, the crude was taken up in EtOAc (3x30 mL) and was decanted to remove the remaining DMF. The oily yellow product was dissolved in H_2O , filtrated to remove *N,N'*-dicyclohexylurea, and purified by flash chromatography on RP-18 ($H_2O/MeOH$ from 1:0 to 4:6). *N*-(9-fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alanine (664.0 mg, 1.225 mmol, 48%, mixture of two racemic diastereomers) was isolated as a white amorphous powder. A small amount of the starting material *N*-(9-fluorenylmethyloxycarbonyl)- α -sulfo- β -alanine (103 mg, 0.263 mmol, 10%) could be recovered from the flash chromatography. 1H NMR ($DMSO-d_6$, 400 MHz) δ (ppm) : 7.88 (2H, d, J = 7.4 Hz), 7.69 (2H, d, J = 7.4 Hz), 7.41 (2H, t, J = 7.4 Hz), 7.33 (2 H, td, J = 1.1-7.4 Hz), 4.69 (br, NH), 4.22-4.21 (3H, 2s), 3.64-3.47 (6H, 2m). ^{13}C NMR ($DMSO-d_6$, 100 MHz) δ (ppm) : 169.5, 169.4, 167.2, 155.8, 143.9, 140.7, 127.7, 127.2, 125.4, 120.1, 65.7, 64.9, 64.8, 64.7, 46.7, 40.3, 40.2, 38.34, 38.27. LRMS (negative mode) (m/z): calcd for $C_{21}H_{21}N_2O_{11}S_2$, 541.06 ($[M-H]^-$). Found 541.07. HPLC (method E), t_R 21.61 min. HPLC (method J), t_R 21.05-21.39 min.

5.1.2.23. *N*-(9-Fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alaninyl- α -sulfo- β -alanine. *N*-(9-Fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alanine (467.2 mg, 0.8620 mmol) and *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) (259.6 mg, 0.8623 mmol, 1.0 equiv) were introduced in anhydrous DMF (11 mL) under argon. DIEA (453 μ L, 2.59 mmol, 3.0 equiv) was added and the mixture was stirred at rt for 17 h. Then the mixture was added dropwise in a stirred solution of NaHCO₃ 6.8% (3.9 mL) at 0 °C containing α -sulfo- β -alanine (160.0 mg, 0.9467 mmol, 1.1 equiv). The mixture was stirred at rt for 39 h before adjusting the pH to 2 (12 N HCl). After concentration, the crude was taken up in Et₂O (3x15 mL) and EtOAc (1x10mL) and was decanted to remove the remaining DMF. The oily yellow product was dissolved in H₂O, filtrated to remove the urea derivative, and purified by flash chromatography on RP-18 (TFA 0.1%/MeOH from 1:0 to 3:7). *N*-(9-fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alaninyl- α -sulfo- β -alanine (292.7 mg, 0.4224 mmol, 49%, mixture of three racemic diastereomers) was isolated as a white amorphous powder. A part of the starting material *N*-(9-fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alanine (92.1 mg, 0.170 mmol, 20%) could be recovered from the flash chromatography. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 8.16 (1H, br), 7.87 (2H, d, *J* = 7.5 Hz), 7.70 (2H, m), 7.40 (2H, t, *J* = 7.3 Hz), 7.33 (2H, m), 5.07 (1H, br), 4.19 (3H, m), 3.70-3.30 (11H, m), 3.13 (2H, m), 1.26-1.25-1.23 (15H, 3s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 169.2, 155.8, 153.5, 143.9, 140.7, 127.6, 127.2, 125.5, 120.1, 65.7, 65.2, 64.9, 64.6, 53.6, 46.6, 41.9, 38.3, 18.1, 16.8, 12.5. LRMS (negative mode) (*m/z*): calcd for C₂₄H₂₆N₃O₁₅S₃, 692.05 ([M-H]⁻). Found 692.07. HPLC (method E), *t*_R 19.58-20.60 min. HPLC (method J), *t*_R 18.32-18.51-18.89 min (activated starting material, *t*_R 22.39-22.57 min).

5.1.3. General Procedure for the Synthesis of Compounds 26 to 39 and 44 to 52

5.1.3.1. Peptide coupling of the linkers on the ethylamine terminated biovectors

Detailed descriptions for the synthesis of each compound are included in the Supporting Information (Table S1).

5.1.3.1.1. Method 1. HOBt (0.2-0.5 equiv), EDCI HCl (1.1 equiv), and the Fmoc protected carboxylic acid terminated linker (1.1 to 1.4 equiv) were dissolved in the solvent and stirred at rt for 15 min. The ethylamine terminated biovector previously dissolved in the solvent was then introduced into the mixture. Portions of EDCI HCl (0.5-1 equiv for one portion) were added to the mixture to complete the conversion of the ethylamine terminated biovector. At the end of the reaction, the mixture was concentrated. A precipitation procedure was performed (H₂O) or the crude was directly purified by flash chromatography to isolate the modified biovector.

5.1.3.1.2. Method 2. The ethylamine terminated biovector, HBTU (1.1 equiv), HOBT (1.1 equiv) and the Fmoc protected carboxylic acid terminated linker (1.0 equiv) were dissolved in DMF. DIEA (3.5 equiv) was introduced and the mixture was stirred at rt for 2 h. At the end of the reaction, the mixture was concentrated and the crude was purified by flash chromatography to isolate the modified biovector.

5.1.3.1.3. Fmoc deprotection of the linkers. The modified biovector was dissolved in DMF and piperidine (0.2-6 equiv) was introduced in the mixture under stirring. At the end of the reaction, the mixture was concentrated. A precipitation procedure was performed (Et₂O) or the crude was directly purified by flash chromatography to isolate the amine terminated biovector.

5.1.3.2. Peptide coupling of the Gd(III) chelates on the amine terminated biovectors.

Detailed descriptions for the synthesis of each compound are included in the Supporting Information (Table S2).

HOBt (0.3-0.5 equiv), EDCI HCl (1.1 equiv), and the carboxylic acid terminated Gd(III) chelate (**Gd-DOTAGA** or **Gd-PCTA12**, 1.0-2.6 equiv) were dissolved in water and stirred at rt for 15 min. The amine terminated biovector, previously dissolved in an organic solvent (DMF or DMSO), was then introduced dropwise into the mixture. The pH was maintained to 6 during the reaction. Portions of EDCI HCl (0.5-1 equiv for one portion) were added to the mixture to complete the conversion of the amine terminated biovector. At the end of the reaction, the mixture was concentrated. If a lysine or an arginine moiety was contained on the linker of the CA, a deprotection step to remove the boc or the Pbf protection group respectively was necessary. For both cleavages, the protected CA was dissolved in TFA and stirred for 1-2 h. After concentration of the reaction mixture, cold water was added to the crude and the pH was quickly adjusted to 6 (10 N NaOH). Finally, a precipitation procedure was performed (Et₂O and/or EtOAc) and/or the crude was purified on reverse phase flash chromatography (RP-2 or RP-18) to isolate the final CA which was lyophilized before storage.

5.1.3.3. Characterisations

5.1.3.3.1. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*_α-(9-fluorenylmethyloxycarbonyl)-*N*_ε-*tert*-butyloxycarbonyl]-*L*-lysine]]-benzothiazole. ¹H NMR (DMSO-*d*₆, 400 MHz) δ(ppm) : 8.13 (1H, br), 7.96 (1H, s), 7.88 (2H, d, *J* = 7.5 Hz), 7.80 (3H, m), 7.71 (2H, m), 7.60 (1H, s), 7.40 (2H, m), 7.34 (2H, t, *J* = 7.5 Hz), 7.05 (1H, dd, *J* = 2.0, 9.0 Hz), 6.81 (2H, d, *J* = 9.1 Hz), 6.70 (1H, br), 4.25 (2H, m), 4.07 (2H, t, *J* = 5.6 Hz), 4.00 (1H, m), 3.50 (3H, m), 3.00 (6H,

s), 2.89 (2H, m), 1.58 (2H, m), 1.42 (13H, m). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 172.7, 166.0, 156.3, 155.9, 152.2, 148.8, 144.2, 141.1, 135.6, 128.4, 127.9, 127.4, 125.7, 122.8, 120.8, 120.5, 116.0, 122.2, 106.2, 77.7, 67.3, 65.9, 55.1, 47.1, 40.0, 38.6, 32.1, 29.6, 28.6, 23.2. HRMS (m/z): calcd for $\text{C}_{43}\text{H}_{50}\text{N}_5\text{O}_6\text{S}$, 764.3476 ($[\text{M}+\text{H}]^+$). Found 764.3487.

5.1.3.3.2. 2-(4-N,N-dimethylaminophenyl)-6-[ethoxyamido-[N ϵ -tert-butyloxycarbonyl]-L-lysine]]-benzothiazole (31). ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm) : 8.13 (1H, br), 7.80 (3H, m), 7.60 (1H, s), 7.05 (1H, d, $J = 7.5$ Hz), 6.80 (2H, d, $J = 9.0$ Hz), 6.70 (1H, br), 4.10 (2H, t, $J = 5.6$ Hz), 3.50 (2H, t, $J = 5.6$ Hz), 3.20 (1H, m), 3.00 (6H, s), 2.85 (2H, m), 1.59-1.25 (15H, m). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 175.3, 165.9, 156.8, 155.9, 152.3, 148.8, 135.6, 128.4, 122.8, 120.8, 115.9, 112.2, 106.1, 77.7, 67.3, 54.7, 40.1, 38.4, 34.8, 29.8, 28.6, 22.9. HRMS (m/z): calcd for $\text{C}_{28}\text{H}_{40}\text{N}_5\text{O}_4\text{S}$, 542.2796 ($[\text{M}+\text{H}]^+$). Found 542.2783. HPLC (method A), t_{R} 4.34 min. HPLC (method G), t_{R} 28.17 min.

5.1.3.3.3. 2-(4-N,N-dimethylaminophenyl)-6-[ethoxyamido-[N α -(9-fluorenylmethyloxycarbonyl)-N ϵ -tert-butyloxycarbonyl]-L-lysine]]-benzoxazole. ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm) : 8.11 (1H, br), 7.87 (6H, m), 7.71 (3H, m), 7.42 (2H, m), 7.35 (2H, t, $J = 7.5$ Hz), 6.94 (1H, dd, $J = 2.0, 9.0$ Hz), 6.85 (2H, d, $J = 9.1$ Hz), 6.71 (1H, br), 4.27 (2H, m), 4.07 (2H, m), 4.00 (1H, m), 3.48 (1H, m), 3.38 (2H, m), 3.03 (6H, s), 2.87 (2H, m), 1.60 (2H, m), 1.5-1.24 (13H, m). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 172.7, 163.0, 156.6, 156.3, 155.9, 152.5, 151.1, 144.2, 141.1, 136.2, 128.5, 128.0, 127.4, 125.7, 122.5, 120.5, 119.8, 113.2, 112.1, 96.9, 77.7, 67.5, 66.0, 55.0, 47.1, 40.0, 38.6, 32.1, 29.6, 28.6, 23.2. HRMS (m/z): calcd for $\text{C}_{43}\text{H}_{50}\text{N}_5\text{O}_7$, 748.3705 ($[\text{M}+\text{H}]^+$). Found 748.3707. HPLC (method A), t_{R} 7.72 min. HPLC (method G), t_{R} 44.53 min.

5.1.3.3.4. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*_ε-*tert*-butyloxycarbonyl]-*L*-lysine]]-benzoxazole (32). ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 8.09 (1H, br), 7.90 (2H, d, *J* = 9.0 Hz), 7.56 (1H, d, *J* = 8.7 Hz), 7.36 (1H, d, *J* = 2.4 Hz), 6.95 (1H, dd, *J* = 2.4, 8.7 Hz), 6.85 (2H, d, *J* = 9.0 Hz), 6.70 (1H, br), 4.08 (2H, t, *J* = 5.6 Hz), 3.50 (2H, t, *J* = 5.6 Hz), 3.41 (1H, m), 3.03 (6H, s), 2.89 (2H, m), 1.56 (2H, m), 1.50-1.25 (13H, m). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 175.9, 163.0, 156.6, 155.9, 152.5, 151.2, 136.2, 128.5, 119.2, 113.6, 113.2, 112.1, 96.9, 77.7, 67.5, 55.0, 40.2, 40.0, 38.2, 35.2, 29.8, 28.6, 23.0. HRMS (*m/z*): calcd for C₂₈H₄₀N₅O₅, 526.3024 ([M+H]⁺). Found 526.3016. HPLC (method A), *t*_R 4.13 min.

5.1.3.3.5. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*_α-(9-fluorenylmethyloxycarbonyl)-*N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine]]-benzothiazole. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 8.19 (1H, br), 7.88 (2H, d, *J* = 7.6 Hz), 7.82 (2H, d, *J* = 8.9 Hz), 7.78 (1H, d, *J* = 8.9 Hz), 7.72 (2H, dd, *J* = 4.7, 7.1 Hz), 7.60 (1H, d, *J* = 2.4 Hz), 7.40 (2H, t, *J* = 7.3 Hz), 7.31 (2H, t, *J* = 7.4 Hz), 7.04 (1H, dd, *J* = 2.4, 8.9 Hz), 6.82 (2H, d, *J* = 8.9 Hz), 4.23 (3H, m), 4.02 (3H, m), 3.48 (2H, m), 3.01 (6H, s), 2.91 (2H, s), 2.55-2.45 (2H, m), 2.48 (3H, s), 2.41 (3H, s), 1.98 (3H, s), 1.70-1.20 (4H, m), 1.37 (6H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 172.9, 165.4, 161.3, 157.4, 156.0, 155.9, 151.9, 148.4, 143.7, 141.2, 140.7, 137.2, 135.1, 131.4, 128.0, 127.6, 127.0, 125.3, 124.3, 122.3, 120.4, 120.0, 116.2, 115.6, 111.8, 105.7, 86.2, 66.9, 65.6, 54.3, 46.6, 42.7, 42.4, 39.5, 38.2, 29.3, 28.2, 24.0, 18.9, 17.5, 12.2. HRMS (*m/z*): calcd for C₅₁H₅₈N₇O₇S₂, 944.3834 ([M+H]⁺). Found 944.3834. HPLC (method A), *t*_R 8.19 min. HPLC (method G), *t*_R 47.77 min.

5.1.3.3.6. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine]]-benzothiazole (**33**). ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 8.26 (1H, br), 7.80 (3H, m), 7.62 (1H, d, *J* = 2.4 Hz), 7.06 (1H, d, *J* = 8.9 Hz), 6.81 (2H, d, *J* = 8.9 Hz), 4.08 (2H, m), 3.50 (2H, m), 3.38 (1H, m), 3.00 (6H, s), 2.94 (2H, s), 2.55-2.47 (2H, m), 2.48 (3H, s), 2.42 (3H, s), 1.99 (3H, s), 1.70-1.20 (4H, m), 1.40 (6H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 172.9, 165.5, 162.3, 157.4, 155.8, 151.9, 148.4, 137.3, 135.2, 131.4, 128.1, 124.3, 122.4, 120.4, 116.3, 115.6, 111.8, 105.8, 86.3, 66.9, 53.8, 42.5, 39.5, 38.1, 31.2, 28.3, 25.2, 19.0, 17.6, 12.3. We notice signals of C24 and C18 are not visible (expected around 141 ppm and 43 ppm respectively). HRMS (*m/z*): calcd for C₃₆H₄₈N₇O₅S₂, 722.3153 ([M+H]²⁺). Found 722.3163. HPLC (method A), *t*_R 4.91 min. HPLC (method G), *t*_R 30.79 min.

5.1.3.3.7. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*_α-(9-fluorenylmethyloxycarbonyl)-tri-[*N*_ε-(*tert*-butyloxycarbonyl)-*L*-lysine]]]-benzothiazole. HRMS (*m/z*): calcd for C₆₅H₉₀N₉O₁₂S, 1220.6435 ([M+H]⁺). Found 1220.6439. HPLC (method A), *t*_R 8.76 min.

5.1.3.3.8. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[tri-[*N*_ε-(*tert*-butyloxycarbonyl)-*L*-lysine]]]-benzothiazole (**44**). HRMS (*m/z*): calcd for C₅₀H₈₀N₉O₁₀S, 998.5743 ([M+H]⁺). Found 998.5748. HPLC (method A), *t*_R 5.70 min. HPLC (method C), *t*_R 7.88 min. HPLC (method G), *t*_R 31.83 min.

5.1.3.3.9. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-tri-[*N*_α-(9-fluorenylmethyloxycarbonyl)-*N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine]]-benzothiazole. HRMS (*m/z*): calcd for C₈₉H₁₁₃N₁₅NaO₁₅S₄, 1782.7316 ([M+Na]⁺). Found 1782.7282. HPLC (method A), *t*_R 9.32 min. HPLC (method G), *t*_R 52.85 min.

5.1.3.3.10. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-tri-[*N*_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*L*-arginine]]-benzothiazole (**45**). HRMS (*m/z*): calcd for C₇₄H₁₀₃N₁₅NaO₁₃S₄, 1560.6635 ([M+Na]⁺). Found 1560.6606. HPLC (method B), *t*_R 6.57 min. HPLC (method G), *t*_R 35.16 min.

5.1.3.3.11. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*-(9-fluorenylmethyloxycarbonyl)- α -sulfo- β -alanine]]-benzothiazole. ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 8.06 (1H, m), 7.87 (2H, d, *J* = 7.3 Hz), 7.81 (2H, d, *J* = 8.0 Hz), 7.78 (1H, d, *J* = 9.7 Hz), 7.66 (2H, d, *J* = 7.2 Hz), 7.59 (1H, s), 7.39 (2H, t, *J* = 7.4 Hz), 7.30 (2H, t, *J* = 7.5 Hz), 7.06 (2H, m), 6.81 (2H, d, *J* = 8.0 Hz), 4.19 (3H, m), 4.03 (2H, t, *J* = 5.6 Hz), 3.49 (5H, m), 3.01 (6H, s). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 167.7, 165.5, 155.9, 155.7, 151.9, 148.4, 143.8, 140.7, 135.2, 128.1, 127.6, 127.1, 125.3, 122.4, 120.1, 115.7, 111.9, 105.9, 67.0, 65.6, 64.9, 46.7, 40.2, 38.2. HRMS (*m/z*): calcd for C₃₄H₃₅N₃O₁₀S₂, 709.1758 ([M+H]⁺). Found 709.1751. HPLC (method E), *t*_R 35.28 min. HPLC (method J), *t*_R 29.73 min.

5.1.3.3.12. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-(α -sulfo- β -alanine)]-benzothiazole (**49**). ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) : 7.83 (1H, d, *J* = 8.9 Hz), 7.81 (2H, t, *J* = 9.0 Hz), 7.64 (1H, d, *J* = 2.6 Hz), 7.08 (1H, d, *J* = 8.9 Hz), 6.81 (2H, d, *J* = 9.0 Hz), 4.06 (2H, m, *J* = 5.6 Hz), 3.70-3.15 (5H, m), 3.05 (4H, br), 3.01 (6H, s), 2.80 (4H, br), 1.50 (2H, br). ¹³C NMR (DMSO-*d*₆, 100 MHz) δ (ppm) : 168.1, 165.5, 155.8, 151.9, 148.5, 135.1, 128.1, 122.4, 120.5, 115.7, 111.8, 106.0, 67.0, 66.2, 45.6, 40.6, 38.2, 24.9, 23.6. HRMS (*m/z*): calcd for C₂₀H₂₅N₄O₅S₂, 465.1261 ([M+H]⁺). Found 465.1259. HPLC (method A), *t*_R 4.34 min. HPLC (method J), *t*_R 21.29 min.

5.1.3.3.13. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-[*N*-(9-fluorenylmethyloxycarbonyl)- α -sulfo- β -alaninyl- α -sulfo- β -alaninyl- α -sulfo- β -alanine]]-benzothiazole. ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm) : 8.16 (3H, br), 7.81 (5H, m), 7.68 (3H, m), 7.39 (2H, m), 7.33 (2H, m), 7.08 (1H, m), 6.79 (2H, d, $J = 9.0$ Hz), 4.19 (3H, m), 4.04 (2H, m), 3.80-3.30 (17H, m), 3.12 (6H, qd, $J = 4.3$ Hz), 3.00 (6H, s), 1.28 (45H, m). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 167.81, 167.76, 167.4, 167.2, 167.0, 166.6, 165.5, 155.9, 155.8, 151.9, 148.4, 143.9, 140.6, 135.2, 128.1, 127.6, 127.2, 125.5, 122.3, 120.6, 120.0, 115.7, 111.9, 105.9, 66.8, 66.6, 65.2, 65.0, 64.6, 64.5, 65.7, 64.7, 53.6, 46.6, 41.9, 41.3, 39.5, 38.0, 18.1, 16.7, 12.5. HRMS (negative mode) (m/z): calcd for $\text{C}_{41}\text{H}_{45}\text{N}_6\text{O}_{15}\text{S}_4$, 987.1675 ($[\text{M}-\text{H}]^-$). Found 987.1639. HPLC (method E), t_R 27.48 min. HPLC (method J), t_R 23.68-23.85 min.

5.1.3.3.14. 2-(4-*N,N*-dimethylaminophenyl)-6-[ethoxyamido-(α -sulfo- β -alaninyl- α -sulfo- β -alaninyl- α -sulfo- β -alanine)]-benzothiazole (**50**). ^1H NMR (DMSO- d_6 , 400 MHz) δ (ppm) : 8.06 (2H, br), 7.82 (6H, m), 7.67 (1H, s), 7.10 (1H, d, $J = 8.8$ Hz), 6.80 (2H, d, $J = 8.9$ Hz), 4.06 (2H, m), 3.85-3.10 (11H, m), 3.00 (14H, m), 1.63 (8H, br), 1.53 (4H, br). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ (ppm) : 167.2, 167.1, 165.9, 165.5, 155.9, 151.9, 148.5, 135.2, 128.1, 122.4, 120.5, 115.7, 111.9, 106.0, 65.0, 64.6, 64.5, 64.34, 64.26, 61.64, 61.59, 43.8, 40.1, 39.5, 38.2, 22.2, 21.7. HRMS (negative mode) (m/z): calcd for $\text{C}_{26}\text{H}_{32}\text{N}_6\text{NaO}_{13}\text{S}_4$, 787.0813 ($[\text{M}-2\text{H}+\text{Na}]^-$). Found 787.0785. HPLC (method E), t_R 21.31-21.67 min. HPLC (method J), t_R 17.28-17.49 min.

5.1.3.3.15. **26**. HRMS (m/z): calcd for $\text{C}_{36}\text{H}_{40}\text{GdN}_7\text{NaO}_9\text{S}$, 927.1741 ($[\text{M}+\text{Na}]^+$). Found 927.1743. HPLC (method A), t_R 4.16 min. HPLC (method G), t_R 26.34 min.

5.1.3.3.16. **27**. HRMS (m/z): calcd for $\text{C}_{36}\text{H}_{40}\text{GdN}_7\text{NaO}_{10}$, 911.1970 ($[\text{M}+\text{Na}]^+$). Found 911.1975. HPLC (method A), t_R 4.03 min. HPLC (method G), t_R 25.64 min.

5.1.3.3.17. **28.** HRMS (m/z): calcd for $C_{36}H_{46}GdN_7Na_2O_{10}S$, 486.1026 ($[M+2Na]^{2+}$). Found 486.1020. HPLC (method A), t_R 4.74 min. HPLC (method G), t_R 30.08 min.

5.1.3.3.18. **29.** HRMS (m/z): calcd for $C_{36}H_{46}dN_7Na_2O_{11}$, 478.1140 ($[M+2Na]^{2+}$). Found 478.1141. HPLC (method C), t_R 4.78 min. HPLC (method G), t_R 29.43 min.

5.1.3.3.19. **30.** HRMS (m/z): calcd for $C_{36}H_{47}GdN_6Na_2O_{10}$, 463.6190 ($[M+2Na]^{2+}$). Found 463.6187. HPLC (method A), t_R 4.02 min. HPLC (method G), t_R 22.68 min.

5.1.3.3.20. **34.** HRMS (m/z): calcd for $C_{42}H_{52}GdN_9Na_2O_{10}S$, 539.1295 ($[M+2Na]^{2+}$). Found 539.1293. HPLC (method A), t_R 3.26 min. HPLC (method G), t_R 22.55 min.

5.1.3.3.21. **35.** HRMS (m/z): calcd for $C_{42}H_{52}GdN_9Na_2O_{11}$, 531.1409 ($[M+2Na]^{2+}$). Found 531.1412. HPLC (method A), t_R 3.18 min. HPLC (method G), t_R 21.96 min.

5.1.3.3.22. **36.** HRMS (m/z): calcd for $C_{42}H_{53}GdN_{11}NaO_{10}S$, 542.1413 ($[M+H+Na]^{2+}$). Found 542.1409. HPLC (method A), t_R 3.27 min. HPLC (method G), t_R 22.17 min.

5.1.3.3.23. **37.** HRMS (m/z): calcd for $C_{42}H_{59}GdN_9NaO_{11}S$, 539.1591 ($[M+H+Na]^{2+}$). Found 539.1582. HPLC (method A), t_R 4.08 min. HPLC (method E), t_R 24.28-25.25 min. HPLC (method G), t_R 24.67-25.40 min.

5.1.3.3.24. **38.** HRMS (m/z): calcd for $C_{42}H_{58}GdN_9NaO_{12}$, 1061.3338 ($[M+Na]^+$). Found 1061.3349. HPLC (method A), t_R 3.73-3.92 min. HPLC (method G), t_R 24.41-25.23 min.

5.1.3.3.25. **39.** HRMS (m/z): calcd for $C_{42}H_{58}GdN_{11}Na_2O_{11}S$, 564.1536 ($[M+2Na]^{2+}$). Found 564.1532. HPLC (method C), t_R 3.91-3.99 min. HPLC (method E), t_R 24.53-25.08 min. HPLC (method G), t_R 25.01-25.44 min.

5.1.3.3.26. **46.** HRMS (m/z): calcd for $C_{54}H_{78}GdN_{19}O_{12}S$, 687.2514 ($[M+2H]^{2+}$). Found 687.2515. HPLC (method C), t_R 2.32 min. HPLC (method G), t_R 20.63 min.

5.1.3.3.27. **47.** HRMS (m/z): calcd for $C_{54}H_{84}GdN_{13}O_{13}S$, 656.2631 ($[M+2H]^{2+}$). Found 656.2632. HPLC (method A), t_R 2.72 min. HPLC (method E), t_R 20.47-20.66-20.80 min. HPLC (method F), t_R 13.67-14.05-14.31-14.82 min. HPLC (method G), t_R 19.05-19.25-19.35 min. HPLC (method K), t_R 8.51-8.76-9.07-9.45 min.

5.1.3.3.28. **48.** HRMS (m/z): calcd for $C_{54}H_{84}GdN_{19}O_{13}S$, 698.2723 ($[M+2H]^{2+}$). Found 698.2729. HPLC (method A), t_R 2.80 min. HPLC (method E), t_R 21.12 min. HPLC (method G), t_R 20.83 min.

5.1.3.3.29. **51.** HPLC (method E), t_R 22.81 min. HPLC (method J), t_R 18.75-18.84 min.

5.1.3.3.30. **52.** HRMS (negative mode) (m/z): calcd for $C_{45}H_{58}GdN_{10}O_{22}S_4$, 458.7290 ($[M-3H]^{3-}$). Found 458.7273. HPLC (method E), t_R 18.79 min. HPLC (method J), t_R 16.15 min.

5.1.4. *Quantification of free Gd^{3+} content*

Quantification of the non-chelated Gd^{3+} , called the free Gd^{3+} , was performed using a colorimetric test with Arsenazo III. The glassware was previously cleaned with ethylenediaminetetraacetic acid (EDTA). The samples were prepared in a triethanolamine 1 M/nitric acid 0.5 M 2:3 pH 7.85 buffer containing Arsenazo III ($4 \cdot 10^{-6}$ M). The free Gd^{3+} concentration in the samples should not exceed $2 \cdot 10^{-6}$ M. The solutions were shaken for 30 min before measuring the absorbance (654 nm, $\epsilon = 34500 \text{ M}^{-1} \cdot \text{cm}^{-1}$). A standard curve with $GdCl_3$ was used to determine the concentration of free Gd^{3+} .

5.1.5. Quantification of total Gd^{3+} content

Gd^{3+} content in the final compounds was measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Optima 3300 RL, Perkin) or by inductively coupled plasma-mass spectroscopy (ICP-MS) (Elan 6100 DRC plus, PerkinElmer) depending on the concentration of the sample. For this purpose, nitric acid 65% (4.5 mL) was added to the gadolinium containing sample (0.5 mL). The mixture was heated at 80 °C for 8 h, allowing a complete dissolution of the Gd chelates. After the mineralization step, the sample was dissolved in MilliQ water to a final concentration of nitric acid at 32.5%. Total Gd^{3+} concentration was quantified by ICP-AES or ICP-MS using a set of calibration standards ranking from 0.1 mg/L to 5 mg/L of Gd^{3+} with Yttrium as internal standard prepared in nitric acid 32.5% (RF power at 1500W, Gd = 342.257 nm, Y = 324,227 nm), or ranking from 0.1 µg/L to 1200 µg/L of Gd^{3+} with Indium as internal standard prepared in nitric acid 6.5% (RF power at 1300W, $^{157}Gd^{+}$, $^{115}In^{+}$) respectively.

5.2. Relaxivity measurements

The free Gd^{3+} presents in the CA samples was measured before the relaxivity and biological studies. When free Gd^{3+} content exceeded 1% of the total Gd^{3+} contained in the sample, a correction on the relaxivity values obtained from the experiment was applied. Subtraction of the relaxation rate induced by the free Gd^{3+} ($1/T_{i(Gd)}$, $i = 1,2$) to the measured relaxation rate ($1/T_{i(mes)}$) gave the intrinsic CA relaxivity (r_{CA}) (Equation 1).

$$r_{CA} = (1/T_{i(mes)} - 1/T_{i(Gd)}) / ([Gd^{3+}]_{(total)} - [Gd^{3+}]_{(free)})$$

Equation 1. Correction applied on the measured relaxivity of the CAs containing more than 1% free Gd^{3+} .

Water proton relaxation measurements were performed with a Bruker Minispec PC-20 relaxometer (Bruker, Wissembourg, France) at 20 & 60 MHz (0.47 & 1.5 T), 37 °C, pH 7.4, on solutions with a concentration of Gd(III) chelates ranging from 0.1 to 5 mM in three different media (sterile water / ion cocktail (1 M chloride, 25 mM carbonate, 0.5 mM phosphate and 0.2 mM citrate) / Seronorm Human™). Spin–lattice relaxation times, T_1 , and spin-spin relaxation times, T_2 , were measured by inversion–recovery and CPMG method respectively. r_1 and r_2 were respectively calculated from the slope of the regression line of $1/T_1$ or $1/T_2$ against concentration with a least-squares method.

5.3. Biological assays

5.3.1. Binding assays on $A\beta_{1-42}$ aggregates

The solid form of peptide $A\beta_{1-42}$ was gently dissolved in 10 mM sodium phosphate, 1 mM EDTA, pH 7.4 sterile buffer (50 μ M). Aliquots (200 μ L) were frozen and stored at -20 °C until their use. An aliquot of peptide was thawed and incubated for 36-42 h at 37 °C under gentle and constant shaking to induce the aggregation. The aggregated peptides were diluted with the buffer to 20 μ M. The aggregated peptides (20 μ M, 50 μ L), the CA (40 μ L, DMSO, concentration range 0.3-1000 nM) and the [125 I]IMPY (1 nM, 50 μ L, ethanol 10%) were diluted with ethanol 10% (860 μ L) and the mixture was incubated at rt for 3 h under gentle agitation. The mixture was then filtered through GF/B filters by using a Brandel M24 Harvester. The filters were washed with

ethanol 10% (2 x 3 mL) and were collected to measure the radioactivity with a gamma-counter Cobra II. The amount of radioactivity retained on filter was determined through a control experiment without aggregated peptide. The software GraphPad Prism 4.02 was used to plot the specific binding of [¹²⁵I]IMPY against the logarithm concentration of the CA to determine the IC₅₀ (quadruplicate). The K_i of IMPY (homologous competition) was calculated from the IC₅₀, using the following equation: $K_d = IC_{50} - L$. The K_i of the CAs was calculated from the IC₅₀, using the following equation: $K_i = IC_{50} / [1 + [\text{radioligand}] / K_d]$.

5.3.2. Binding assays on human Alzheimer brain homogenates

Postmortem brain tissues were obtained from four AD patients (Braak stage 4–6) and four age-matched controls aged 67–90 at autopsy. *Pre-* and *postmortem* consents were obtained from next of kin, and brains were removed at autopsy, sectioned fresh and frozen at –70 °C. The cerebellum and the affected frontal cortex of AD patients together with the corresponding regions of the controls were dissected out. Homogenates were then prepared in PBS, pH 7.4, at the concentration of approximately 100 mg wet tissue/mL (motor-driven glass homogenizer with setting of 6 for 30 s). The homogenates were aliquoted (1 mL portions) and stored at –70 °C for 3–6 months without loss of binding signal. Human Alzheimer brain homogenates (25 µg/well) were incubated at 37 °C for 120 min with [¹²⁵I]IMPY (42 nM) in the absence or presence of the tested CA. The non-specific binding was measured in the presence of IMPY (1 µM). Following incubation, the samples were rapidly filtered through GF/B glass fiber filters using a Packard cell harvester to separate the free radiolabeled ligand from that bound to the homogenates. The filters were dried and the retained radioactivity was quantified in a scintillation counter (Topcount,

Packard) using a scintillation cocktail (Microscint 0, Packard). The data were treated with GraphPad Prism 4.02 (duplicate).

5.3.3. BBB crossing assays

These experiments were performed by the company PreCeM (LEMM group at iBiTech-S Institute, CEA Saclay, Gif-sur-Yvette, France). The *in vitro* BBB model used was constituted of a co-culture of rat primary brain capillary endothelial cells and rat glial cells supported by a polycarbonate filter (0.4 μm). A solution of the CA in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 μM) was introduced in the donor compartment (blood) and incubated at 37 °C for 1 h. The BBB permeation was determined by the quantification of the CA migration from the donor to the receiver (brain) compartments. The CA concentration was measured with ICP-MS. The experiments were performed in triplicate. The integrity and permeation of the BBB model was confirmed using sucrose and vinblastine.

Supporting Information

Tables which summarize the conditions used to synthesize each CA and synthon, as well as the HPLC and GC analytical methods used, the dose-response curves of the CA affinity study, the longitudinal and transversal relaxivity parameters measured at 20 MHz and 60 MHz in water, ion cocktail and seronorm, and the results of the BBB permeability study. The ^1H and ^{13}C NMR signal attribution is also included.

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