ABSTRACT: The GdAAZTA (AAZTA = 6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid) complex represents a platform of great interest for the design of innovative MRI probes due to its remarkable magnetic properties, thermodynamic stability, kinetic inertness, and high chemical versatility. Here, we detail the synthesis and characterization of new derivatives functionalized with four amino acids with different molecular weights and charges: L-serine, L-cysteine, L-lysine, and L-glutamic acid. The main reason for conjugating these moieties to the ligand AAZTA is the in-depth study of the chemical properties in aqueous solution of model compounds that mimic complex structures based on polypeptide fragments used in molecular imaging applications. The analysis of the $^1$H NMR spectra of the corresponding Eu(III)-complexes indicates the presence of a single isomeric species in solution, and measurements of the luminescence lifetimes show that functionalization with amino acid residues maintains the hydration state of the parent complex unaltered ($q = 2$). The relaxometric properties of the Gd(III) chelates were analyzed by multinuclear and multifrequency NMR techniques to evaluate the molecular parameters that determine their performance as MRI probes. The relaxivity values of all of the novel chelates are higher than that of GdAAZTA over the entire range of applied magnetic fields because of the slower rotational dynamics. Data obtained in reconstituted human serum indicate the occurrence of weak interactions with the proteins, which result in larger relaxivity values at the typical imaging fields. Finally, all of the new complexes are characterized by excellent chemical stability in biological matrices over time, by the absence of transmetallation processes, or the formation of ternary complexes with oxyanions of biological relevance. In particular, the kinetic stability of the new complexes, measured by monitoring the release of Gd$^{3+}$ in the presence of a large excess of Zn$^{2+}$, is ca. two orders of magnitude higher than that of the clinical MRI contrast agent GdDTPA.

INTRODUCTION

Magnetic resonance imaging (MRI) represents a powerful tool in diagnostic medicine and preclinical biomedical research that enables visualization of anatomical images with high spatial and temporal resolution in a noninvasive way. The image contrast in MRI depends on the difference in concentration and, primarily, in the relaxation properties of the water proton nuclei present in different body tissues. Although the natural contrast is superb, it is not always sufficient to provide accurate diagnostic information, which can be enhanced with the use of exogenous contrast agents (CAs).\(^1\)\(^-\)\(^3\) These are low-molecular-weight paramagnetic chelates able to improve the contrast-to-noise ratio of MR images by efficiently shortening the relaxation times of nearby water protons. CAs are inorganic probes administered intravenously, which when distributed in the bloodstream extravasate nonspecifically into tissues and are eliminated rapidly through the kidneys. The most used probes in MR diagnostic imaging are Gd-based contrast agents (GBCAs), in which the metal ion reaches its most stable coordination number (CN = 9) by binding octadentate linear or macrocyclic ligands and one water molecule ($q = 1$).\(^4\)-\(^6\) Despite the excellent properties characterizing such probes, including high thermodynamic stability, kinetic inertness and rapid clearance, their ability to enhance relaxation is significantly lower than that theoretically predicted. Such issue motivates the search for new classes of CAs with improved efficacy, which can be administered in lower doses, to reduce costs and minimize the possible risks associated with long-term accumulation of Gd$^{3+}$.

Over the last decades, several Gd-complexes with promising features as potential MRI CAs have been developed, among which the 6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid Gd(III)-chelate (GdAAZTA) has stood out for its...
excellent properties.\textsuperscript{7} In fact, despite being a heptadentate ligand, AAZTA is able to bind Gd(III) with stability comparable to the commercial octadentate analogues. In addition, the two sites available to complete the coordination number nine are occupied by two water molecules ($q = 2$), which impart an increased relaxation enhancement capacity to the chelate.\textsuperscript{8}

The efficacy with which a CA relaxes the proton nuclei of nearby water molecules is described by relaxivity ($r_1$), defined as the increase of the water protons’ longitudinal relaxation rate ($R_1$) per millimolar unit of concentration of the paramagnetic ion. At clinical magnetic field strengths (1–7 T), $r_1$ mainly depends on the molecular tumbling rate ($\tau_b$) of the chelate, hydration state ($q$), the average lifetime ($\tau_m$) of the coordinated water molecules, and the electronic relaxation parameters ($\Delta^2$ and $\tau_s$) of the metal ion.\textsuperscript{1,2} Interestingly, in clinical fields, GdAAZTA shows relaxivity values higher than that measured for clinically used CAs ($r_1 = 6.6 \text{ mM}^{-1} \text{s}^{-1}$ and $r_1 \sim 5 \text{ mM}^{-1} \text{s}^{-1}$ at 298 K and 1.5 T, respectively).\textsuperscript{9} Most importantly, unlike many $q = 2$ complexes, GdAAZTA is characterized by high thermodynamic stability and kinetic inertness toward dissociation, transmetalation, and transferrin-mediated demetallation.\textsuperscript{9,10} Another important property of the complex lies in the dynamics of exchange of the two water molecules coordinated to the metal center. We have recently shown that the two inner-sphere waters have substantially different residence lifetimes as a direct consequence of the structural characteristics of the complex.\textsuperscript{8} Such hydration molecules are located at different positions in the coordination polyhedron of the complex, where the one occupying the more sterically hindered capping position exchanges $\sim 6$ times faster than that residing closer to the metal center.\textsuperscript{8}

Such favorable properties have promoted the development of several GdAAZTA derivatives with improved relaxivity,\textsuperscript{11} which have found interesting applications in preclinical MRI studies.\textsuperscript{12} In particular, the evidence that high molecular weight, slowly tumbling molecules provide greater $r_1$ values in the 0.5–1.5 T range of magnetic field strengths has driven the design of several GdAAZTA macromolecular systems, where the chelate is either covalently bound to large substrates or forms noncovalent macromolecular adducts. For instance, substantial efforts have been made in developing (i) dimeric,\textsuperscript{13,14} multimeric, or dendrimeric derivatives alone,\textsuperscript{15,16} or grafted to PEGylated mesoporous silica nanoparticles,\textsuperscript{17} and (ii) lipophilic GdAAZTA complexes capable of assembling in supramolecular aggregates, such as micelles or liposomes,\textsuperscript{18} which can achieve relaxivity values up to 10 times higher than those of the monomeric species. Remarkably, lipophilic GdAAZTA derivatives have also shown the ability to form high-affinity supramolecular complexes with human serum albumin (HSA) if functionalized with suitable aliphatic groups\textsuperscript{19,20} or with bile acid-like side chains.\textsuperscript{21} This confers to the chelate a prolonged lifetime in the bloodstream and high relaxivity properties, which make it a suitable blood pool agent. In addition, GdAAZTA has also been conjugated with a wide range of targeting vectors capable of specific interactions with biomolecules other than HSA, with the aim of developing contrast agents for molecular imaging applications. Such biochemically targeted probes include GdAAZTA coupled with lipids targeting the liver fatty acid binding protein (L-FABP),\textsuperscript{22} with peptidomimetics interacting with integrin $\alpha$,$\beta_3$ expressed in cancers cells,\textsuperscript{23} and with fibrin targeting peptides\textsuperscript{16,24} as pathological biomarkers.

Although much effort has been made in developing high-molecular-weight GdAAZTA derivatives with improved relaxometric performances as potential probes for molecular imaging, little is known about the structural changes that could improve their effectiveness. In fact, maximum relaxivity values are not only achievable by slowing down molecular tumbling motions but also by simultaneously fine-tuning the molecular dynamics, exchange dynamics, and electronic parameters, the latter being closely related to the structural features of the metal complex. These bio-conjugated structures are often difficult to characterize in detail with high- and low-resolution NMR techniques because of their intrinsic complexity associated with their high molecular weight and poor solubility.

For these reasons, the incorporation of low-molecular-weight amino acids into the AAZTA ligand, capable of mimicking the influence of polypeptide fragments on the relaxometric properties of more complex structures, could represent a general and effective approach prior to the synthesis of derivatives for molecular imaging applications. It is well established that the introduction of peripheral functionalities in the ligand can affect the molecular parameters.
that govern the relaxivity of the corresponding Gd(III) chelates. Of course, the conjugation to more complex biomolecules can further change a few relaxometric parameters (e.g., the exchange rate of the metal-bound water molecules and the molecular tumbling rate), but we hypothesize that only the functional groups closest to the metal center are able to influence other key properties of GdAAZTA, such as electronic relaxation and hydration state.

Here, we present the synthesis of a novel library of AAZTA-amino acid derivatives (AAZTA-aa) and a comprehensive NMR analysis of the structural and relaxometric properties of their corresponding Gd-chelates. Four AAZTA-aa derivatives were synthesized, each functionalized with residues of different charge, steric hindrance, and hydrophilicity (i.e., L-serine, L-cysteine, L-lysine, and L-glutamic acid) was successfully obtained, as summarized in Scheme S1. The syntheses were carried out by a solid-phase synthetic approach, following a standardized Fmoc protocol and starting from a Fmoc-Rink Amide MBHA resin. The Fmoc-protected amino acid was separately anchored to the resin using PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate) as the activating agent and DIPEA (N,N-diisopropylethamine) as the base. After removal of the Fmoc protecting group, the chelator was conjugated by reacting overnight (tBu)₄-AAZTA-C₄-COOH in the presence of PyBOP and DIPEA. After cleavage from the resin, the final purification was carried out by semi-preparative reversed-phase chromatography, obtaining the final products with a purity of over 95% and an overall yield between 17 and 20% (Figures S1–S4). The solid-phase approach enables a single purification step, avoiding extractions and manual direct-phase chromatography otherwise needed for solution chemistry synthetic procedures. The comparable yields of the four AAZTA-aa derivatives demonstrate the reproducibility of the protocol, which can be generally applied to synthesize different AAZTA-aa conjugates, with high chemical purities, ready to chelate trivalent metal ions used for magnetic resonance applications.

The purity of the ligands was confirmed by UPLC-MS analysis (Figures S1–S4) and high-resolution 1D and 2D NMR spectroscopy (Figures S5–S12).

**RESULTS AND DISCUSSION**

**Synthesis of the Ligands and Complexes.** A novel library of AAZTA-amino acid derivatives (AAZTA-aa) and a comprehensive NMR analysis of the structural and relaxometric properties of their corresponding Gd-chelates. Four AAZTA-aa derivatives were synthesized, each functionalized with residues of different charge, steric hindrance, and hydrophilicity (i.e., L-serine (AAZTA-Ser), L-cysteine (AAZTA-Cys), L-lysine (AAZTA-Lys), and L-glutamic acid (AAZTA-Glu) (Scheme 1)), which are directly linked to the coordination cage of the ligand, exploiting the side chain of the chelator which is not involved in the metal complexation (Scheme 1).

The choice of such systems is based on the evidence that small GBCAs-amino acid conjugates can be accumulated in tumor cells in higher amounts than their benign counterparts, as already demonstrated for GdDOTA-like complexes functionalized with glutamine residues.

**High-Resolution NMR and Luminescence Studies on EuAAZTA-Glu.** To obtain structural information on the
paramagnetic GdAAZTA-aa complexes, high-resolution NMR studies were performed on the EuAAZTA derivatives. The $^1$H NMR spectra were acquired at 300 K (Figure S13), showing a single set of resonances similar to what was observed for EuAAZTA. The $^1$H NMR profiles do not change significantly in the 278–300 K range. This indicates that the introduction of amino acid functionalities does not alter the structure of the AAZTA coordination cage and, most importantly, suggests the presence of a single isomer in solution.

To assess the hydration state ($q$) of the GdAAZTA-aa complexes in solution, luminescence measurements were carried out on the EuAAZTA-Glu chelate and compared with that of the EuAAZTA complex (Figure S14). Luminescence decay profiles were measured over time on the two Eu(III)-complexes dissolved in pure H$_2$O and in D$_2$O under excitation at 370 nm (Figure S14). The so-obtained decay curves were fitted by a single exponential function to obtain the fluorescence lifetimes. Both complexes share a bis-hydrated coordination sphere ($q = 2$) as calculated by comparing the fluorescence lifetimes measured in H$_2$O and D$_2$O (Table S1).

We assume that all of the GdAAZTA-aa family members have the same number of inner-sphere water molecules, since they share an identical coordination cage.

**pH Dependency of Proton Relaxivity.** The chemical stability of the GdAAZTA-aa complexes was evaluated by measuring the $^1$H relaxivity ($r_1$) as a function of pH. The $r_1$ values were recorded on ~2 mM aqueous solutions of the GdAAZTA-aa complexes in the pH range between 2.0 and 11.0 (Figure S15) at 32 MHz and 298 K. The experimental results show constant values of $r_1$ over the entire range of pH investigated, indicating excellent chemical stability of the metal complexes and an unchanged hydration state. It is particularly significant that $r_1$ does not decrease at basic pH, as commonly observed in $q = 2$ Gd-complexes, as this indicates the lack of formation of ternary complexes with the dissolved carbonate, which involves the displacement of the metal-bound water molecules. Furthermore, we can safely conclude that the protonation state of the residues does not affect the pH dependency of relaxivity.

**$^1$H NMRD Profiles.** To obtain information on the molecular and dynamic parameters responsible for the relaxation properties of GdAAZTA-aa derivatives, a detailed analysis of the field-dependent relaxivity profiles was performed.

Nuclear magnetic relaxation dispersion (NMRD) profiles were acquired on ~2 mM aqueous solutions of the GdAAZTA-Cys, GdAAZTA-Ser, GdAAZTA-Lys, and GdAAZTA-Glu complexes at neutral pH, by measuring relaxivity ($r_1$) in the $^1$H Larmor frequency range between 0.01 and 120 MHz and at three different temperatures (283, 298, and 310 K) (Figure 1). The profiles were analyzed by Solomon–Bloembergen–Morgan and Freed equations that describe the inner- and the outer-sphere contributions to the relaxation, respectively (see ESI for more details on the equations used). The NMRD profiles of the chelates display very similar behavior to each other and to GdAAZTA, which is typical of low-molecular-weight systems with fast molecular tumbling rates. The profiles are characterized by a low-field plateau between 0.01 and 1 MHz, followed by a single dispersion at about 4 MHz, and a high-field plateau between 20 and 120 MHz. For all of the GdAAZTA-aa derivatives, the relaxivity values measured over the entire Larmor frequency range decrease with increasing temperature, as expected for small-sized complexes characterized by fast exchange regimes ($T_{1M} > r_M$). Under these conditions, $T_{1M} (\sim \mu s)$ is longer than the mean residency time of the metal-bound water molecule ($r_M \sim 100$ ns) and limits the proton relaxivity, influencing its temperature dependence. In turn, $T_{1M}$ depends on the correlation time ($\tau_c$), which is largely dominated by the rotational dynamics ($\tau_R \sim ps$) for small complexes. Consequently, at rising temperatures, the increased rotational dynamics (shorter $\tau_R$ and $\tau_c$) causes an increase in $T_{1M}$ and, therefore, a decrease in relaxivity. Such a trend is clearly visible in the variable temperature (VT) profiles of $r_1$ acquired on the GdAAZTA-aa chelates, in the temperature range between 283 and 320 K, at 32 MHz (Figure S16). In the VT-NMR profiles, $r_1$ decreases with increasing temperatures following a mono-exponential decay, as expected for systems in fast exchange conditions. However, the analysis of these curves does not allow obtaining quantitative information on the exchange dynamics that characterizes the two inner-sphere water molecules. In fact, in the fast exchange regime, $r_M$ does not influence $r_1$, which is dominated by $T_{1M}$ instead. In such conditions, $r_1$ largely depends on $q$ and $\tau_R$ as a first approximation. Therefore, the 2-fold relaxivity enhancement measured at 32 MHz for the GdAAZTA-aa complexes with respect to the GdAAZTA chelate is attributable to the lengthening of $\tau_R$ associated with an increase of their molecular weight (MW). As expected, a linear correlation of relaxivity on the molecular size is observed by plotting $r_1$ vs MW for the GdAAZTA-aa derivatives, with the exception of GdAAZTA-Ser, which slightly deviates from linearity (Figure S17).

**$^{17}$O NMR Measurements.** Detailed information on the inner-sphere water exchange dynamics of the GdAAZTA-aa complexes was obtained by measuring the $^{17}$O transverse relaxation rates ($R_2 = 1/T_2$) and chemical shift ($\Delta\delta$) of the bulk water as a function of temperature (280–350 K) at high field (11.74 T) (Figures 2 and S18). The $^{17}$O-$R_2$ profiles of the four GdAAZTA-aa complexes show similar asymmetrical “bell” shapes characterized by maxima around 300 K and unusual...
trends at temperatures below 285 K, resembling that observed for GdAAZTA. Such behavior is attributable to the presence of two Gd-bound water molecules occupying different positions of the coordination polyhedron with different bond lengths and therefore characterized by different rates of exchange. The hydration water closer to the metal center is in an intermediate exchange regime with the bulk and generates a 17O-R1 maximum at ~300 K. The more labile and distant from Gd(III) water ligand has a faster rate of exchange (kex = 1/τM), and is responsible for the 17O-R1 increase observed below 285 K. As an example, the distinct contribution of the two water molecules to the 17O-R2 profile of GdAAZTA-Glu is emphasized in Figure S19.

In principle, the presence of multiple isomers in solution featuring different water exchange rates could also account for such an unusual trend of the 17O-R1 profiles, as already observed for other Gd(III) chelates.37,38 However, such hypothesis is ruled out by the presence of a single set of signals in the variable temperature NMR spectra of the EuAAZTA-aa complexes (Figure S13).20

Quantitative Analysis of the 1H NMRD and 17O NMR Data. A global analysis of the experimental 1H NMRD and 17O NMR data was performed simultaneously. Solomon–Bloembergen–Morgan31–34 and Freed35 equations that describe the inner- and the outer-sphere contributions to the relaxation were used for the analysis of the NMRD profiles. Swift–Connick equations were used for the analysis of the 17O NMR data.39 The equations have been modified to account for the different contributions of the two water ligands, which are characterized by different residence lifetimes (τM1 and τM2), associated exchange enthalpies (ΔH1M1 and ΔH2M2), and hyperfine scalar coupling constants (A(O/H) and A(C/H)).

To aid the fitting procedure, some of the structural and dynamic parameters affecting 1H T1 and 17O T1 relaxations were set to the values reported for the GdAAZTA complex.8 In particular, the number of water ligands (q = 2, according to the photoluminescence analyses reported above), the distance between the inner-sphere water protons and Gd(III) (r = 3.0 Å), the closest distance between an outer-sphere water molecule and the paramagnetic center (a = 4.0 Å), the relative diffusion coefficient of the outer-sphere water molecules and the complex at 298 K (D = 2.24 × 10^{-5} cm^2 s^{-1}), the activation energy for the diffusion coefficient (E_D = 20 kJ mol^{-1}), and the activation energy of zero-field splitting modulation (E_0 = 1.0 kJ mol^{-1}) were taken into account.

An excellent fit of the 1H NMRD and 17O NMR data of the GdAAZTA-aa complexes (Figures 1 and 2) was obtained with the parameters listed in Tables 1 and S2. The parameters describing the electron spin relaxation of the paramagnetic metal (i.e., the correlation time associated with the modulation of the zero-field splitting (ZFS) interaction τν and the square of the mean ZFS energy Δ2) assume values that are similar among the different GdAAZTA-aa complexes and in agreement with those reported for GdAAZTA. This indicates that AAZTA functionalization with amino acids of different nature does not alter the symmetry of the coordination cage and, therefore, the electronic relaxation properties of the metal ion in the Gd-complex.

On the other hand, variations in the exchange dynamics of the two metal-bound water molecules are observable among the different GdAAZTA-aa derivatives and with respect to GdAAZTA. In particular, the water exchange dynamics is modulated by the steric hindrance and charge of the amino acid side chains. In fact, the substitution of the methyl group with more sterically hindered amino acid functionalities reduces the residence time of the more labile water ligand with respect to GdAAZTA, while increasing the residence lifetime of that closer to Gd(III), as observed for the negatively charged GdAAZTA-aa complexes (τM1 (GdAAZTA-aa) = 22.5–24.8 ns and τM2 (GdAAZTA-aa) = 190–245 ns and τM2 (GdAAZTA) = 169 ns) (Tables 1 and S2). Such a trend can be attributed to the increased steric crowding at the water binding site that favors the dissociation of the more labile water molecule and consequently stabilizes the less hindered one, as already observed for the negatively charged LnAAZTA complexes. For the latter systems, the lanthanide contraction along the series increases the steric compression and leads to the loss of the more labile water molecule between Ho and Er. This phenomenon is associated with a remarkable stabilization of the residual water ligand, whose water exchange rate drops by 2–3 orders of magnitude toward the end of the series.26 On the other hand, in the case of the neutrally charged GdAAZTA-Lys complex, the τM1 reduction and τM2 elongation expected from the increased steric crowding are mitigated by more favorable water–metal interactions compared to the negatively charged complexes (Table 1). In fact, the neutral charge of the chelate promotes stronger electrostatic water–metal interactions and causes a slowdown of the overall water exchange kinetics. The residency time of the rapidly exchanging water ligand is higher than that found for the negatively charged complexes and comparable to that of GdAAZTA. Moreover, the dynamics of the slower exchanging water ligand is reduced with respect to GdAAZTA and to the negatively charged complexes, with the exception of GdAAZTA-Glu (Table 1). Surprisingly, the GdAAZTA-Glu

Table 1. Parameters Obtained from the Simultaneous Analysis of 17O NMR and 1H NMRD Data Acquired on the GdAAZTA-aa Complexes

|                  | GdAAZTA-Ser | GdAAZTA-Cys | GdAAZTA-Lys | GdAAZTA-Glu | GdAAZTA-aa |
|------------------|-------------|-------------|-------------|-------------|------------|
| τM1 (298 K) (ns) | 245         | 245         | 245         | 245         | 245        |
| τM2 (298 K) (ns) | 190         | 190         | 190         | 190         | 190        |
| ΔH1M1 (kJ mol^{-1}) | 20.0       | 20.0       | 20.0       | 20.0       | 20.0       |
| ΔH2M2 (kJ mol^{-1}) | 29.2       | 29.2       | 29.2       | 29.2       | 29.2       |
| τν (ps)          | 23.3        | 23.3        | 23.3        | 23.3        | 23.3       |
| τν (ps)          | 30.0        | 30.0        | 30.0        | 30.0        | 30.0       |

aParameter fixed during the fitting procedure. bData from ref 8.
complex is characterized by the coordinated water molecule residing for the longest time on the metal center despite its bulkier and negatively charged side chain.

For all of the GdAAZTA-aa complexes, the hydration equilibrium of the more labile water molecule has an enthalpy value \( \sim 1.5 \) times lower than that associated with the other (\( \Delta H_{M1} \sim 20 \text{ kJ mol}^{-1} \) and \( \Delta H_{M2} \sim 30 \text{ kJ mol}^{-1} \)), as also observed for GdAAZTA.8

The values of the rotational correlation times obtained from the data fits are sensibly longer than that of GdAAZTA (\( \tau_R = 115-140 \text{ ps} \) vs \( \tau_R = 74 \text{ ps} \), respectively), as expected for complexes with increased size and molecular mass. The decrease of the rotational dynamics of the GdAAZTA-aa chelates mainly accounts for their remarkable relaxivity enhancement compared to GdAAZTA. The \( \tau_R \) value calculated for GdAAZTA-Cys allows excluding a possible dimerization of the complex under these experimental conditions, promoted by the oxidation of the -SH group. Interestingly, the highest relaxivity value is measured for GdAAZTA-Ser chelate (\( \tau_R = 12.4 \text{ m}^{-1} \text{ s}^{-1} \) at 60 MHz, 298 K) despite having the lowest molecular mass. This can be attributed to possible hydrogen bonds between the polar OH-group of the serine side chain and water molecules, which generate a second sphere contribution to the relaxation that was considered during the data analysis. The experimental data are in excellent agreement with the presence of a single second sphere water molecule (\( q^{\ominus} = 1 \)) at a distance of 3.5 Å from the paramagnetic center (Table S2). The so-obtained best-fitting curves correctly describe the experimental data. The electronic parameters (Table S2). The so-obtained best-fitting curves correctly describe the experimental data. The electronic parameters obtained from the fitting procedure for the four analyzed complexes (Tables 1 and S2) are in excellent agreement with those reported in previous studies, pointing out that this type of functionalization keeps the coordination cage of the metal ion unchanged.

**Kinetic Inertness of the GdAAZTA-aa Complexes.** An important feature of GdAAZTA is the good kinetic inertia, a very relevant aspect for bioimaging applications. To obtain a qualitative evaluation of the inertness of the GdAAZTA-aa derivatives toward the transmetallation processes, the rates of metal ion displacement of the chelates were determined by challenging the complexes with 25 equiv of \( \text{Zn}^{2+} \) (pH 6.30, 310 K) and monitoring the \( G^+ \) release by recording \( T_1 \) values at 10 MHz as a function of time (Figure 3). Such a test is widely adopted in the literature for a preliminary evaluation of the inertness of the MRI probes in vitro.40 In fact, as the concentration of \( \text{Zn}^{2+} \) in the blood is relatively high (55–125 mM), it may favor the displacement of a significant amount of Gd\(^{3+}\). Other potential competitors in vivo, including Cu\(^{2+}\), Ca\(^{2+}\), or Fe\(^{3+}\), can be neglected, as Cu\(^{2+}\) concentration is low (1–10 mM); Ca\(^{2+}\) ions typically show a low affinity constant with organic ligands, and Fe\(^{3+}\) is not easily available for transmetallation.34

Furthermore, the same measurements were performed on the GdAAZTA, GdHPD03A, and GdDTPA complexes as a comparative benchmark. The pseudo-first-order constants of the transmetallation reaction, obtained from the fitting of the experimental data (Experimental section), are reported in Table 2. The kinetic constants found for the GdAAZTA-aa complexes are comparable with those of GdAAZTA, indicating that the ligand functionalization with amino acid residues does not alter the kinetic stability of the Gd(III)-chelate. In addition, the excellent kinetic stability of the GdAAZTA-aa complexes is comparable to (GdHPD03A) or ca. 2 order of magnitude higher (GdDTPA) than that of clinically approved MRI contrast agents.40

**Stability of the GdAAZTA-aa Complexes in Biological Matrices.** In vitro stability studies of the GdAAZTA-aa complexes in a simulated physiological environment were carried out to evaluate their stability for *in vivo* applications. The lyophilized human serum Seronorm was used to simulate the biological matrix. \(^1\)H NMR relaxometric studies were carried out on aqueous solutions of the GdAAZTA-aa complexes in the presence of reconstituted human serum (Figures 4 and S19) over 10 days to check the chemical stability of the complexes (Figure 4). In addition, \(^1\)H NMRD profiles acquired on the GdAAZTA-aa complexes in pure water and in Seronorm were compared to assess the occurrence of possible interactions between the paramagnetic complexes and the biological matrix (Figures 4 and S20).

The profiles show shape and amplitude that are significantly different in the frequency range between 10 MHz and 120 MHz and nearly identical at lower frequency values. Unlike GdAAZTA, the GdAAZTA-aa chelates show a broad hump in the high-fields range in the profiles collected in the presence of Seronorm, which is absent in pure water. For all GdAAZTA derivatives, the \( R_1 \) peak in the NMRD profiles in Seronorm represents a notable increase, corresponding to an enhancement of 45.3, 22.6, and 39.7% (32 MHz) for GdAAZTA-Cys, GdAAZTA-Ser, and GdAAZTA-Glu, respectively, and as much as 84.2% for GdAAZTA-Lys (52 MHz). Considering the high stability characterizing the GdAAZTA-aa complexes, such
In conclusion, the herein synthetic procedure can be exploited to obtain different amino acid-chelator conjugates with high chemical purity, ready for complexation with gadolinium or other trivalent metal ions of interest in magnetic resonance applications (e.g., paraCEST). The use of a solid-phase synthesis approach enables the performance of just one purification step, avoiding extractions and manual direct-phase chromatography, as expected by solution chemistry. The synthesis of four different AAZTA-amino acid derivatives with comparable overall yields demonstrates the reproducibility of the synthetic protocol, which can be generally applied for the generation of different chelator-amino acid conjugates. In-depth NMR analyses were performed on the AAZTA-aa ligands and their Gd(III) and Eu(III) complexes to understand their structural, relaxometric, and stability properties. High-resolution NMR studies showed the presence of a single isomer in solution and highlighted structural analogy with the EuAAZTA complex, indicating the retention of the structural properties of the coordination cage upon introduction of amino acid functionalities. The hydration number of the GdAAZTA-aa complexes is also retained, as demonstrated by luminescence lifetimes data. The two coordinated water molecules exhibit significantly different exchange rates, where the one closer to the metal resides for a longer time at the metal center, while the other exchanges up to 10 times faster. This behavior, which is consistent with that recently reported for the GdAAZTA complex, has been accessed by the simultaneous analysis of the water $^1$H longitudinal and $^17$O transverse relaxation rates and chemical shift variations as a function of the magnetic field strength and temperature, respectively. The resulting picture shows that, for negatively charged complexes, the increased steric compression on the coordination cage accelerates the exchange dynamics of the more labile water molecule while slowing down the dynamics of that more tightly bound to the metal center with respect to GdAAZTA. Such behavior is amplified the higher the steric hindrance and the negative charge are, as observed for GdAAZTA-Glu that possesses the highest exchange rate for the most labile water molecule and the lowest for the least labile one. However, for the neutrally charged complex GdAAZTA-Lys, the same effect caused by increased steric compression is mitigated by the more favorable electrostatic interactions between the Gd(III) and the water ligands. In addition, from a detailed analysis of the molecular parameters controlling the relaxation properties of GdAAZTA-aa derivatives, it emerges that their increased size proportionally reduces their rotational dynamics in solution, thus increasing their relaxivity values in clinical fields. GaAAZTA-Ser represents the only exception, showing a significant second sphere contribution to relaxation. In the case of our new complexes, the incorporation of the amino acid groups in the ligand structure does not alter the hydration state and the electronic parameters of the parent GdAAZTA. This is an important information because it allows for making reliable predictions on the relaxivity of more complex bio-conjugated structures.

The kinetic and chemical stability of the GdAAZTA-aa complexes were preliminarily examined to evaluate their potential applicability for in vivo studies. The kinetic inertness of the GdAAZTA-aa complexes is comparable to that of GdAAZTA and to the macrocyclic clinical contrast agent GdHPDO3A. Finally, the GdAAZTA derivatives show excellent chemical stability in biological matrices over time, with the absence of metal ion release phenomena or the formation of ternary complexes with oxygen xenon anions, which further highlights the remarkable chemical stability of these complexes. Therefore, the collected data support the suitability for in vivo preclinical applications of these novel GdAAZTA-aa complexes.

**CONCLUSIONS**

In conclusion, the herein synthetic procedure can be exploited to obtain different amino acid-chelator conjugates with high chemical purity, ready for complexation with gadolinium or other trivalent metal ions of interest in magnetic resonance applications (e.g., paraCEST). The use of a solid-phase synthesis approach enables the performance of just one purification step, avoiding extractions and manual direct-phase chromatography, as expected by solution chemistry. The synthesis of four different AAZTA-amino acid derivatives with comparable overall yields demonstrates the reproducibility of the synthetic protocol, which can be generally applied for the generation of different chelator-amino acid conjugates. In-depth NMR analyses were performed on the AAZTA-aa ligands and their Gd(III) and Eu(III) complexes to understand their structural, relaxometric, and stability properties. High-resolution NMR studies showed the presence of a single isomer in solution and highlighted structural analogy with the EuAAZTA complex, indicating the retention of the structural properties of the coordination cage upon introduction of amino acid functionalities. The hydration number of the GdAAZTA-aa complexes is also retained, as demonstrated by luminescence lifetimes data. The two coordinated water molecules exhibit significantly different exchange rates, where the one closer to the metal resides for a longer time at the metal center, while the other exchanges up to 10 times faster. This behavior, which is consistent with that recently reported for the GdAAZTA complex, has been accessed by the simultaneous analysis of the water $^1$H longitudinal and $^17$O transverse relaxation rates and chemical shift variations as a function of the magnetic field strength and temperature, respectively. The resulting picture shows that, for negatively charged complexes, the increased steric compression on the coordination cage accelerates the exchange dynamics of the more labile water molecule while slowing down the dynamics of that more tightly bound to the metal center with respect to GdAAZTA. Such behavior is amplified the higher the steric hindrance and the negative charge are, as observed for GdAAZTA-Glu that possesses the highest exchange rate for the most labile water molecule and the lowest for the least labile one. However, for the neutrally charged complex GdAAZTA-Lys, the same effect caused by increased steric compression is mitigated by the more favorable electrostatic interactions between the Gd(III) and the water ligands. In addition, from a detailed analysis of the molecular parameters controlling the relaxation properties of GdAAZTA-aa derivatives, it emerges that their increased size proportionally reduces their rotational dynamics in solution, thus increasing their relaxivity values in clinical fields. GaAAZTA-Ser represents the only exception, showing a significant second sphere contribution to relaxation. In the case of our new complexes, the incorporation of the amino acid groups in the ligand structure does not alter the hydration state and the electronic parameters of the parent GdAAZTA. This is an important information because it allows for making reliable predictions on the relaxivity of more complex bio-conjugated structures.

The kinetic and chemical stability of the GdAAZTA-aa complexes were preliminarily examined to evaluate their potential applicability for in vivo studies. The kinetic inertness of the GdAAZTA-aa complexes is comparable to that of GdAAZTA and to the macrocyclic clinical contrast agent GdHPDO3A. Finally, the GdAAZTA derivatives show excellent chemical stability in biological matrices over time, with the absence of metal ion release phenomena or the formation of ternary complexes with oxygen xenon anions, which further highlights the remarkable chemical stability of these complexes. Therefore, the collected data support the suitability for in vivo preclinical applications of these novel GdAAZTA-aa complexes.

**MATERIALS AND METHODS**

All Fmoc (Fluorenylmethyloxycarbonyl)-protected amino acids, Fmoc-Rink Amide MBHA resin, and PyBOP were purchased from Novabiochem (Darmstadt, Germany), Sigma-Aldrich (Darmstadt, Germany) and Iris Biotech (Marktreuthitz, Germany). All other reagents were purchased from Sigma-Aldrich (Darmstadt, Germany). All solvents were purchased from VWR International (Radnor, USA) and were used without further purification. 6-[[2-(1,1-dimethylethyl)oxy]-2-oxoethyl]amino]-6-(5-carboxypentyl)tetrahydro-1H-1,4-diazepine-1,4(5H)-Diacetic acid N,N’-bis(1,1-dimethylthoxy)-120 MHz) of the GdAAZTA-Lys complex in pure water ($^0$H) and in the presence of Seronorm, (298 K, pH 7.4, [Gd$^{3+}$] = 0.5 mM) over time.

![Figure 4](https://doi.org/10.1021/acs.inorgchem.2c02110)
Briefly, 400 mg of a Fmoc-Rink Amide MBHA resin (loading 0.59 mmol/g) were swelled for 10 min with DMF (N, N-Dimethylformamide). All reaction steps were performed under gentle stirring (35 rpm) and at room temperature. The resin was filtered, and 10 mL of a solution of piperidine 20% in DMF was added to the reactor vessel. After 10 min, the resin was filtered and washed with DMF. Typically, 5 equiv of Fmoc-AA-OH (AA = Ser, Cys, Lys, and Glu), 10 equiv of DIPEA (N,N-Diisopropylethylamine), and 4.5 equiv of PyBOP previously dissolved in DMF (10 mL) were added to the reactor vessel. After 2 h, the resin was filtered and extensively washed with DMF. The resin was filtered, and 10 mL of capping solution (Acetic Anhydride/DIPEA/DMF 1:1:3) was added to the reactor vessel. After 30 min, the resin was filtered and extensively washed with DMF. The resin was filtered, and 10 mL of a solution of piperidine 20% in DMF was added to the reactor vessel. After 30 min, the resin was filtered and washed with DMF. Then, 1.5 equiv of (Fmoc)-AATA-CNCOOH, 3 equiv of DIPEA, and 1.35 equiv of PyBOP previously dissolved in DMF (10 mL) were added to the reactor vessel. The reaction was further stirred overnight. The resin was filtered and extensively washed with DMF, DCM (methylene chloride), and diethyl ether. Then, 10 mL of cleavage solution (Triluoracetic Acid/Trisopropylsilane/H2O 95:2.5:2.5) was added to the reaction vessel, and the reaction was stirred overnight. The crude product was precipitated in cold diethyl ether, and the final purification was achieved by semi-preparative RP-HPLC on a Waters AutoPurification system. Eluent: (A) 0.1% TFA in H2O, (B) 0.1% TFA in CH3CN. Gradient profile: linear gradient from 2 to 20% of B in 7 min, linear gradient from 20 to 100% in 3 min, isocratic at 100% for 1 min. Flow rate: 15 mL/min. The pure product was isolated as a homogeneous peak with a retention time of ca. 4 minutes. The solvent was evaporated in vacuo, and the product was lyophilized from water to give the desired product as a white solid. The purity of the final product was checked by analytical UPLC-MS. Eluent: (A) 0.05% TFA in H2O, (B) 0.05% TFA in CH3CN. Gradient profile: linear gradient from 5 to 50% of B in 7 min, linear gradient from 50 to 100% in 3 min, isocratic at 100% for 3 min; flow rate of 0.4 mL/min and UV detection at 210 nm.

Preparation of the LnAAZTA-aa Complexes. LnAAZTA-aa complexes were prepared by adding 1.1 equiv of LnCl3 salts to an aqueous solution of the AAZTA-aa ligands at pH = 5.5. After the addition, the pH was adjusted to 6.0 with an aqueous solution of NaOH 1 M, and the solution was stirred at room temperature (r.t.) for 12 h. Then, the pH was increased to 10 with 0.1 M NaOH, and the solution was stirred for 3 h to promote the precipitation of the uncomplexed Ln(III) as insoluble hydroxides. The solution was centrifuged (10,000 rpm, 5 min, r.t.); the supernatant was filtered through 0.2 μm filters and neutralized with dilute HCl to separate Ln hydroxides from the solution. The concentration of Ln(III) complexes was evaluated by 1H NMR measurements at 11.7 Tesla, using the well-established bulk magnetic susceptibility method. GdAAZTA-Ser: ESI-MS (m/z): calcd For GdC20H34N2O23 (M + H)+ 975.67; found, 975.63. Preparation of the LnAAZTA-aa Complexes. LnAAZTA-aa complexes were prepared by adding 1.1 equiv of LnCl3 salts to an aqueous solution of the AAZTA-aa ligands at pH = 5.5. After the addition, the pH was adjusted to 6.0 with an aqueous solution of NaOH 1 M, and the solution was stirred at room temperature (r.t.) for 12 h. Then, the pH was increased to 10 with 0.1 M NaOH, and the solution was stirred for 3 h to promote the precipitation of the uncomplexed Ln(III) as insoluble hydroxides. The solution was centrifuged (10,000 rpm, 5 min, r.t.); the supernatant was filtered through 0.2 μm filters and neutralized with dilute HCl to separate Ln hydroxides from the solution. The concentration of Ln(III) complexes was evaluated by 1H NMR measurements at 11.7 Tesla, using the well-established bulk magnetic susceptibility method. GdAAZTA-Ser: ESI-MS (m/z): calcd For GdC20H34N2O23 (M + H)+ 975.67; found, 975.63. Preparation of the LnAAZTA-aa Complexes. LnAAZTA-aa complexes were prepared by adding 1.1 equiv of LnCl3 salts to an aqueous solution of the AAZTA-aa ligands at pH = 5.5. After the addition, the pH was adjusted to 6.0 with an aqueous solution of NaOH 1 M, and the solution was stirred at room temperature (r.t.) for 12 h. Then, the pH was increased to 10 with 0.1 M NaOH, and the solution was stirred for 3 h to promote the precipitation of the uncomplexed Ln(III) as insoluble hydroxides. The solution was centrifuged (10,000 rpm, 5 min, r.t.); the supernatant was filtered through 0.2 μm filters and neutralized with dilute HCl to separate Ln hydroxides from the solution. The concentration of Ln(III) complexes was evaluated by 1H NMR measurements at 11.7 Tesla, using the well-established bulk magnetic susceptibility method. GdAAZTA-Ser: ESI-MS (m/z): calcd For GdC20H34N2O23 (M + H)+ 975.67; found, 975.63. Preparation of the LnAAZTA-aa Complexes. LnAAZTA-aa complexes were prepared by adding 1.1 equiv of LnCl3 salts to an aqueous solution of the AAZTA-aa ligands at pH = 5.5. After the addition, the pH was adjusted to 6.0 with an aqueous solution of NaOH 1 M, and the solution was stirred at room temperature (r.t.) for 12 h. Then, the pH was increased to 10 with 0.1 M NaOH, and the solution was stirred for 3 h to promote the precipitation of the uncomplexed Ln(III) as insoluble hydroxides. The solution was centrifuged (10,000 rpm, 5 min, r.t.); the supernatant was filtered through 0.2 μm filters and neutralized with dilute HCl to separate Ln hydroxides from the solution. The concentration of Ln(III) complexes was evaluated by 1H NMR measurements at 11.7 Tesla, using the well-established bulk magnetic susceptibility method. GdAAZTA-Ser: ESI-MS (m/z): calcd For GdC20H34N2O23 (M + H)+ 975.67; found, 975.63. Preparation of the LnAAZTA-aa Complexes. LnAAZTA-aa complexes were prepared by adding 1.1 equiv of LnCl3 salts to an aqueous solution of the AAZTA-aa ligands at pH = 5.5. After the addition, the pH was adjusted to 6.0 with an aqueous solution of NaOH 1 M, and the solution was stirred at room temperature (r.t.) for 12 h. Then, the pH was increased to 10 with 0.1 M NaOH, and the solution was stirred for 3 h to promote the precipitation of the uncomplexed Ln(III) as insoluble hydroxides. The solution was centrifuged (10,000 rpm, 5 min, r.t.); the supernatant was filtered through 0.2 μm filters and neutralized with dilute HCl to separate Ln hydroxides from the solution. The concentration of Ln(III) complexes was evaluated by 1H NMR measurements at 11.7 Tesla, using the well-established bulk magnetic susceptibility method. GdAAZTA-Ser: ESI-MS (m/z): calcd For GdC20H34N2O23 (M + H)+ 975.67; found, 975.63.
Magnetic susceptibility contribution was subtracted from the 2% isotope enrichment. The transverse relaxation rates were measured by raising the pH from 6.5 to basic values with the addition of 0.1 M HCl.

The complexes, at 32 MHz, 298 K, in the pH range ∼2.1 (br s, 2H), 3.3 (br s, 2H), 3.0−2.0 (br s, 6H), −2.1 (br s, 2H), −7.8 (br s, 2H), −9.5 (br s, 2H).

\(^1\)H NMRD Measurements. 1/T\(_1\)\) 1H Nuclear magnetic relaxation dispersion (NMRD) profiles were acquired with a fast field cycling (FFC) Stelar SMArTracer relaxometer (Stelar s.r.l., Mede, PV, Italy) over a range of proton Larmor frequencies from 9.97 × 10\(^{-3}\) to 10 MHz, with an uncertainty from 1/T\(_1\)\) of ca. 1%. In the range 20−120 MHz proton Larmor frequency were measured with a high-field relaxometer (Stelar) equipped with the HTS-110 3T Metrology cryogen-free superconducting magnet. The NMRD profiles were acquired at three different temperatures (283, 298, and 310 K). The temperature was controlled during the measurements with a Stelar VTC-91 heater airflow equipped with a copper—constantan thermocouple (uncertainty of ±0.1 °C). The real temperature inside the probe was monitored by a Fluke 52k/j digital thermometer (Fluke, Zürich, Switzerland). The data were collected using the standard inversion recovery sequence (16 experiments, 3 scans) with a typical 90° pulse width of 3.5 μs. The reproducibility of the data was within ±0.5%.

Relaxivity measurements of aqueous solutions of the GdAAZTA-aa complexes were performed to gain information on the stability of the Gd\(^{3+}\) complexes. \(r_1\) values were measured for the 2 mM solutions of the complexes, at 32 MHz, 298 K, in the pH range ∼2.0−10.0. \(r_1\) values remain constant in the entire pH range. The pH dependence was measured by raising the pH from 6.5 to basic values with the addition of 0.1 M NaOH and then by lowering it to acid values with the addition of 0.1 M HCl.

\(^1\)H NMRD profiles were acquired on GdAAZTA-aa complexes dissolved in reconstituted human serum (Seronorm) for the stability studies ([Gd\(^{3+}\)] = 0.5 mM, pH = 7.4 and 298 K).

\(^{17}\)O NMR \(T_2\) Measurements. Variable temperature \(^{17}\)O NMR measurements were recorded on a Bruker Avance III spectrometer (11.7 T) equipped with a 5 mm double resonance Z-gradient broadband probe and Bruker BVT-3000 unit for temperature control. The sample was prepared in a 3 mm NMR tube by mixing 188 L of D\(_2\)O and 10 μL of H\(_3\)O\(^+\) (Cambridge Isotope, 2% isotope enrichment). The transverse relaxation rates were calculated from the signal full width at a half-height. The bulk magnetic susceptibility contribution was subtracted from the \(^{17}\)O NMR shift data using the \(^1\)H NMR shifts of the tert-butanol signal as the internal reference. Other details of the instrumentation, experimental methods, and data analysis have been previously reported.\(^3\)

\(^{1}\)H NMR measurements. The one-dimensional \(^1\)H and \(^{13}\)C Nuclear magnetic resonance (NMR) spectra of the AAZTA-aa ligands and of the EuAAZTA-Glu complex in solution were recorded at 298 K with a Bruker Avance III spectrometer equipped with a wide bore 11.7 Tesla magnet. Briefly, 5−10 mM molar samples were prepared by dissolving the samples in isotopically enriched water (D\(_2\)O) for NMR analyses. The 2D COSY spectra acquired on the ligands were collected using a standard phase-insensitive COSY sequence with gradient coherence selection, with 2048 acquired data points in F2, 256 times increments in F1, 16 scans, a 2 s recycle delay, and a spectral window (both F2 and F1) of 10 ppm.

Zn(II) Transmetallation Kinetics. Displacement of Gd(III) by Zn(II) was monitored by monitoring the evolution of free Gd(III) concentration by \(T_1\)-relaxometry. Solutions of the Gd(III) complexes and ZnCl\(_2\)-H\(_2\)O were prepared in a 1:2.5 molar ratio at pH 6.3. The \(r_1\) data were fitted to the below equation\(^4\)

\[r_1 = (r_e - r_m) e^{-K_{obs}t} + r_m\]

Where \(r_e, r_m\) and \(r_m\) are respectively initial relaxivity, at equilibrium, and at time \(t\) of the transmetallation reaction, and \(K_{obs}\) is the pseudo-first-order kinetic constant of the transmetallation reaction.

Luminescence Measurements. Two solutions of 1 mM EuAAZTA-Glu chelate were prepared by dissolving the complex in 1 mL of H\(_2\)O and D\(_2\)O, respectively. Luminescence decays were measured on a Horiba Jobin Yvon Model IBL FL-322 Fluorolog 3 spectrophotofluorometer working in the time-correlated single-photon counting mode (TCSPC) using a SpectraLED (370 nm) for the excitation and monitoring the emission signal of Eu(III) at 365 nm. The signals were collected with an IBH DataStation Hub photon counting module, and the data were analyzed with the DAS6 (Horiba Jobin Yvon IBH) software. The obtained decay curves were then fitted with a mono-exponential decay function to obtain the lifetime of the excited level in H\(_2\)O and D\(_2\)O. The hydration state of the complex was then determined by applying Parker and co-workers’ equation.\(^5\)

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.inorgchem.2c02110.

UPLC-MS and high-resolution NMR spectra of the AAZTA-aa ligands. \(^1\)H NMR spectra of EuAAZTA-aa. Photoluminescence intensity decay curves of EuAAZTA and EuAAZTA-Glu samples. pH and temperature dependence of relaxivity and \(^{17}\)O NMR data of GdAAZTA-aa chelates. NMRD profiles of GdAAZTA-aa in Seronorm matrix (PDF)

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Author Contributions

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Notes

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