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# HIGHLY LUMINESCENT Eu(III) COMPLEXES AS PROBES FOR BIOMOLECULAR SENSING

***Eu(III) complexes can be exploited as probes in the detection of analytes in biological fluids, by means of luminescence. These complexes must be stable in aqueous solution, absorb and efficiently transfer the UV excitation light to the metal ion. We succeeded to obtain this goal by including isoquinoline antenna into the ligand backbone. The luminescence of the complexes significantly increases in the presence of the main analytes of the interstitial extracellular fluid (i.e. hydrogen carbonate, serum albumin and citrate).***

The application of luminescent lanthanide-based compounds became extremely attractive thank to their unique spectroscopic properties: sharp emission bands, broad absorption and excitation spectra, long excited state lifetime **[1]**. The luminescence of lanthanide salts is weak because of the low energy absorption of the metallic ion. Hence, organic chelated complexes of lanthanides are commonly used, where an organic chromophore is used as a sensitizer for lanthanide center luminescence. The antenna chromophore absorbs light with a high molar absorptivity, and transfers the excited state energy to the lanthanide center that consequently emits. This phenomenon is called “antenna effect” **[2]**. The most used candidates in biological imaging are Eu(III) and Tb(III) complexes, due to their high luminescence. Therefore, a suitable chromophore within the structure of the organic ligand is an important task when designing Ln-based complexes for sensing purposes. The tunability of the optical response upon the binding of the analyte to the metal center is often due to the removal of water molecules from the inner coordination sphere. In this case, the system experiences an increase of the overall luminosity due to the decreased efficiency of the non-radiative multiphonon relaxation process caused by the high energy OH vibrations of water.

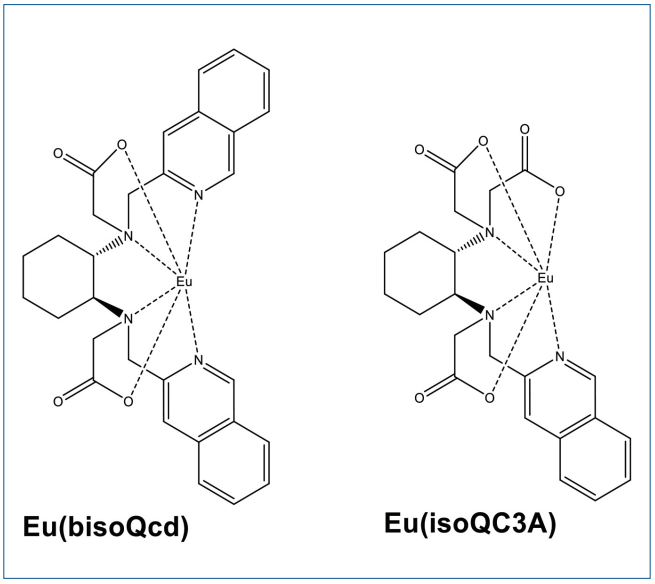


Fig. 1 - Molecular structure of the Eu(III) isoquinoline based complexes

Another important aspect to consider is the selectivity and sensitivity of the optical probe towards biologically relevant biomolecules or anions **[3]**. As far as the optical sensing of important bio-analytes is concerned, hydrogen carbonate ($HCO_3^-$), lactate, citrate and serum albumin proteins (BSA),

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| Reaction | *iso*QC3A | *biso*Qcd [8] | PyC3A [9] | QC3A [9] |
|---|---|---|---|---|
| | | log*Kj* | | |
| L+H⇆HL | 9.43±0.03 | 9.27 | 10.26 | 10.53 |
| HL+H⇆$H_2$L | 7.37±0.07 | 5.86 | 6.33 | 6.29 |
| $H_2$L+H⇆$H_3$L | 3.32±0.09 | 3.43 | 3.67 | 3.60 |
| $H_3$L+H⇆$H_4$L | 2.16±0.12 | 1.62 | 2.01 | 2.81 |
| | | log$\beta$ | | |
| L+Eu⇆EuL | 14.63±0.12 | 10.53 | 15.68 | 12.55 |
| [a]Charges are omitted for simplicity | | | | |

Tab. 1 - Protonation constants (log*Kj*) for the ligand *iso*QC3A and formation constants (log$\beta$) for the complex with Eu(III) at 25 °C and μ=0.1 M NaCl[a]. Additional protonation and formation constant data for comparison with other ligands are also reported

have been widely investigated, since they are among the main species contained in an interstitial extracellular fluid. In order to develop new luminescent Ln-probes for sensing of such relevant biomolecules, here are proposed a family of Eu(III) complexes containing the DACH (1,2-diaminocyclohexane) backbone and a series of antennas differing in charge and steric hindrance at the metal ion **[4-7]**. In this work, the [Eu(*biso*Qcd)] complex and the neutral analogue Eu(*iso*QC3A) (Fig. 1) are tested as luminescent sensors for several bioanalytes present in extracellular fluids. For the latter complex, solution studies were performed to determine the speciation, while for [Eu(*biso*Qcd)] data were obtained previously **[8]**. Structural and spectroscopic information were provided by the analysis of the luminescence spectra, excited states lifetimes and DFT calculations. The performance of the complexes considered here in the luminescent sensing of citrate, $HCO_3^-$ and BSA was assessed by monitoring the Eu(III) emission during titrations of solutions containing either the individual analytes, to obtain the binding constants, or their mixtures to simulate real extracellular environments. Even though some efficient probes based on luminescent lanthanide complexes have been successfully used for the detection of citrate in other biological fluids, our investigated Eu(III) complexes represent the first example of selective optical probes towards citrate in interstitial extracellular fluid-like matrix.

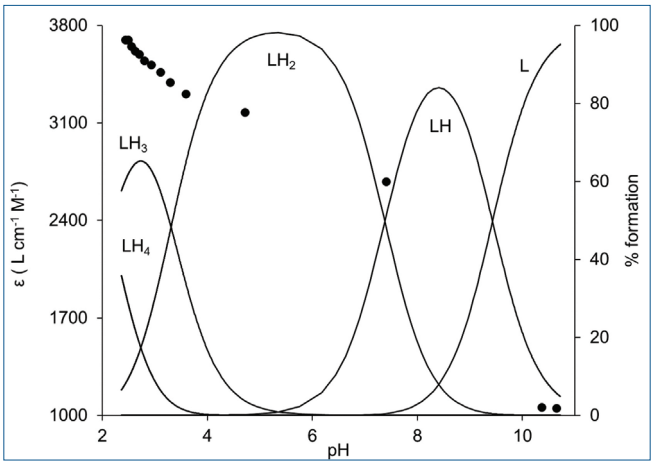


Fig. 2 - Species distribution of the ligand *iso*QC3A (C°L=0.045 mM) with molar absorbance values at λ= 343 nm (●) obtained by acid-base spectrophotometric titration (25 °C, μ=0.1 M NaCl). Charges were omitted for clarity

## Protonation and complex formation

The protonation constants for *iso*QC3A were obtained from the best fit of emf data and are reported in Tab. 1, along with those relative to *biso*Qcd and other ligands containing the DACH backbone. In addition, a spectrophotometric acid-base titration was carried out to evidence the species distribution of the ligand as a function of pH, with attention to molar absorbance ($\varepsilon_\lambda$) variations (Fig. 2). The first protonation constant (log$K_1$=9.43) can be assigned to a tertiary amine, in agreement with those already reported (log*K*~6.9-10.7, depend-

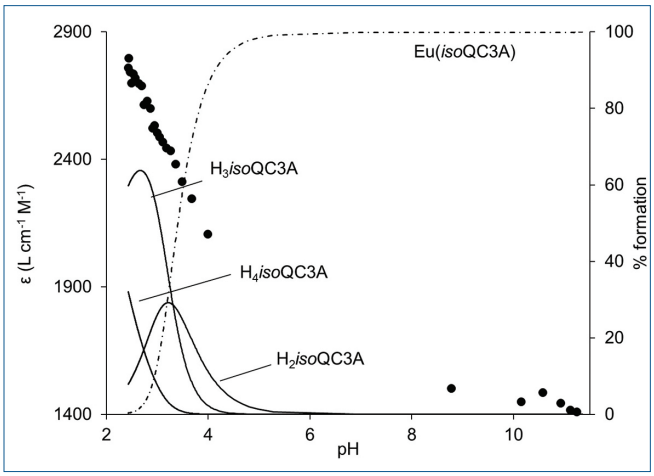


Fig. 3 - Species distribution of the complexes of *iso*QC3A with Eu(III) (M:L ratio 1:1), obtained by acid-base spectrophotometric titration (25 °C, μ=0.1 M NaCl). Molar absorbance at λ = 343 nm (●) are also reported. Charges are omitted for clarity

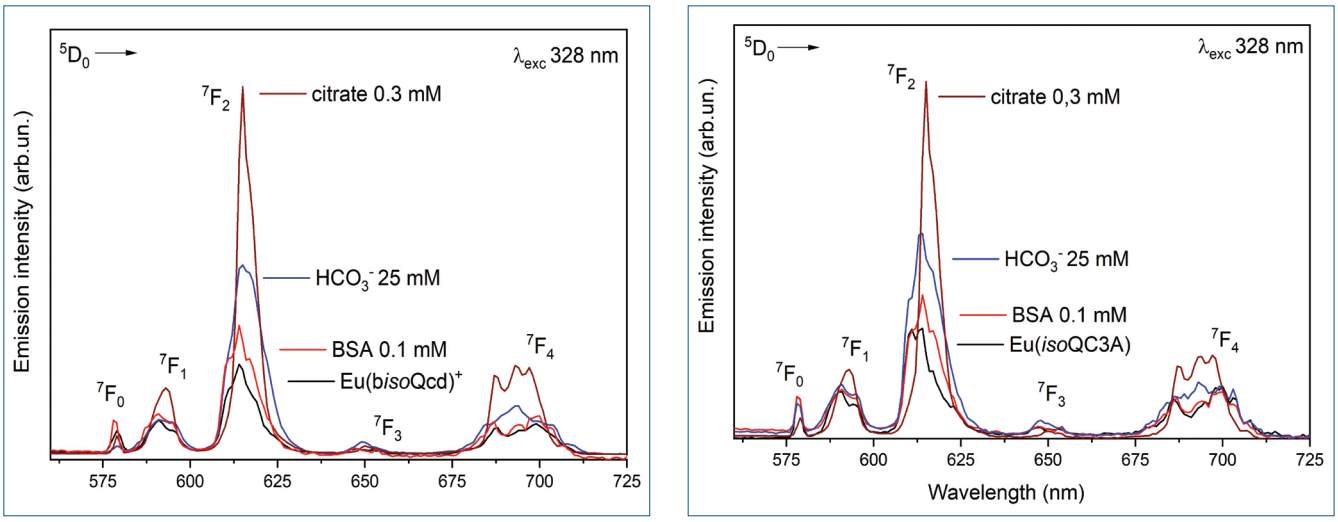


Fig. 4 - Eu(III) luminescence emission spectra upon addition of $HCO_3^-$, BSA and citrate around their typical extracellular concentrations (reported in the picture) for (left) Eu(*biso*Qcd) and (right) Eu(*iso*QC3A) complexes. The concentration of the complexes was 80 μM

ing on the substituents) **[10]**, which indicate the involvement of aliphatic amino group. For isoQC3A the $\varepsilon_\lambda$ is almost constant around pH=10, with following increase due to the formation of bi- and tri-protonated species (log$K_2$=7.37, log$K_3$=3.32). This can be explained by the occurrence of protonation at the isoquinoline moieties, in accordance with the protonation constant value relative to isoquinoline (log$K$=5.46) **[11]**. The last protonation constant found (log$K_4$=2.16) is associated to an acetate group **[12]**. The spectrophotometric data (Fig. 3) relative to the titrations of Eu(III) with *iso*QC3A were best-fitted by admitting only the formation of a EuL complex (Tab. 1). According to this model, at physiological pH=7.4 and 1:1 Eu L molar ratio, the ML species is largely prevalent (~100%). In Fig. 3 the speciation diagram of the Eu(III) *iso*QC3A complex is shown, where the molar absorbance ($\varepsilon_\lambda$) at 343 nm is reported as a function of pH. As expected, the decrease of $\varepsilon_\lambda$ occurs with the formation of the complex. The stability constant is overall similar to those of other triacetate analogues, in specific, higher than that of the Ln(III) complexes with quinoline-substituted ligands, but lower than for the pyridine analogues, due to the increased steric hindrance and less strong interaction of the isoquinoline moieties with respect to the pyridine one.

## Luminescence analysis of the adducts of the Eu(III) complexes with $HCO_3^-$, serum albumin and citrate

The potential of both Eu(III) complexes to serve as probes for the luminescence determination of important analytes that are present in various biological environments were explored. The change of the luminescence emission spectra of Eu(*biso*Qcd) and of Eu(*iso*QC3A) upon titration with each of the main analytes contained in a typical extracellular interstitial fluid was monitored. The complexes show a notable increase of the luminescence intensity in response to the presence of typical concentrations of citrate, $HCO_3^-$ and albumin protein (Fig. 4). Very small or no change in the luminescence spectra are

| | | log$K$ | | |
|---|---|---|---|---|
| | *n* | citrate | $HCO_3^-$ | BSA |
| Eu(*biso*Qcd) | 1 | 4.1±0.3 | - | 3.9±0.2 |
| | 2 | - | 4.6±0.2* | 3.6±0.4‡ |
| Eu(*iso*QC3A) | 1 | 4.1±0.2 | 3.4±0.1 | 4.2±0.6 |

*[Eu(*biso*Qcd)]·$(HCO_3^-)_2$ adduct has been detected
‡[Eu(*biso*Qcd)]·BSA and $[Eu(biso\text{Qcd})]_2$·BSA adducts have been detected

Tab. 2 - Binding constants (log$K$) of the analytes to Eu(III) complexes referred to the reaction: EuL + *n*A ⇆ EuL(A)$_n$

detected when both complexes were titrated with lactate, hydrogen phosphate, sulfate anions, at their typical extra cellular concentrations [L-lactate (2.3 mM), $Na_2HPO_4$ (1.3 mM) and $Na_2SO_4$ (0.6 mM)]. Hence, we conclude that our optical probes are selective towards citrate, $HCO_3^-$ and the albumin protein. From the evolution of the luminescence emission spectra during the titrations it was possible to define the stoichiometry of the adducts formed between the optical probes and the analytes and to calculate the binding constants (Tab. 2) **[8, 13]**.
The values of the binding constants are relatively high and similar for both Eu(III)-complexes and a clear selectivity towards a particular analyte was not observed. By using the observed excited state lifetimes ($\tau_{obs}$) from the Eu(III) $^5D_0$ state (achievable from the decay curve) and the estimation of the $\tau_{rad}$ by means of the Werts equation, we could derive the intrinsic quantum yield of each Eu(III) adduct at its saturation concentration (Tab. 3).
For both complexes, it is interesting to note that all the Eu(III) adducts show higher luminescence efficiency than the related starting complexes. However, among these adducts the ones with citrate exhibits the highest luminescence efficiency. As reported previously both $HCO_3^-$ anion and BSA are capable to displace at least one water molecule from the inner coordination sphere of Eu(III) which increases the luminescence quantum yield by reducing the efficiency of the non-radiative multiphonon relaxation process **[8, 9]**. The flexible citrate anion could be able to coordinate the metal ion in different ways and can therefore remove water molecules from the coordination spheres most efficiently. DFT calculations can be useful to clarify this point: the minimum energy structures of the trans-*O,O* isomers of [Y(*iso*QC3A)cit]$^{3-}$ and [Y(*biso*Qcd)cit]$^{2-}$ with the citrate in all possible coordination modes have been calculated. When the α-hydoxyl and α-carboxylate groups of citrate are coordinated, are energetically most favoured as found in the only reported structure of a citrate adduct of a Eu(III) complex.

| Adduct | $\tau_{obs}$ (ms) | $\tau_{rad}$(ms) | $\phi_{Ln}$(%) |
|---|---|---|---|
| Eu(*biso*Qcd):BSA* | 0.35(1) | 5.38 | 6.5 |
| Eu(*biso*Qcd):$(HCO_3^-)_2$ | 0.46(1) | 3.70 | 12.4 |
| Eu(*biso*Qcd):citrate | 0.83(1) | 3.77 | 22.0 |
| Eu(*iso*QC3A):BSA | 0.33(1) | 5.16 | 6.4 |
| Eu(*iso*QC3A):$HCO_3^-$ | 0.43(1) | 3.71 | 11.6 |
| Eu(*iso*QC3A):citrate | 0.72(1) | 3.47 | 21.0 |

*The presence of $[Eu(biso Qcd)]_2$:BSA adduct is negligible at the working concentration

Tab. 3 - Observed and radiative lifetimes, and intrinsic quantum yield [$\phi_{Ln}$(%)] for the luminescent adducts under investigation

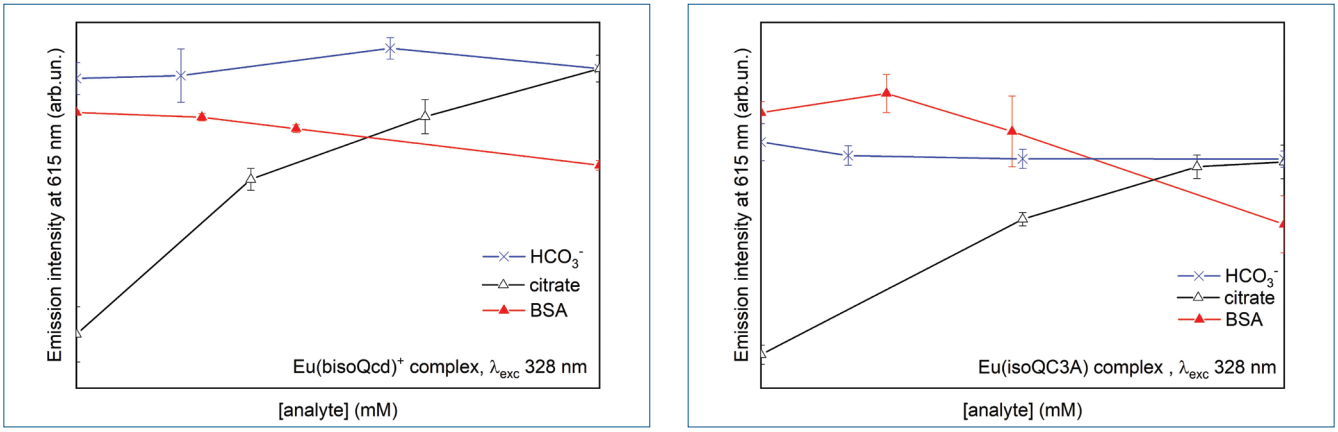


Fig. 5 - Evolution of the Eu(III) emission intensity upon titration (in the presence of interfering species) of (left) Eu(*biso*Qcd) and (right) Eu(*iso*QC3A) with: $HCO_3^-$ (experiment 1, blue line, crosses, 0-25 mM $HCO_3^-$ concentration range; 0.4 mM BSA and 0.3 mM citrate as interferents); BSA (experiment 2, red line, triangle, 0-0.4 mM BSA concentration range; 25 mM $HCO_3^-$ and 0.3 mM citrate as interferents) and citrate (experiment 3, black line, open triangle, 0-0.3 mM citrate concentration range; 25 mM $HCO_3^-$ and 0.4 mM BSA as interferents). The concentration of the complex was 80 μM

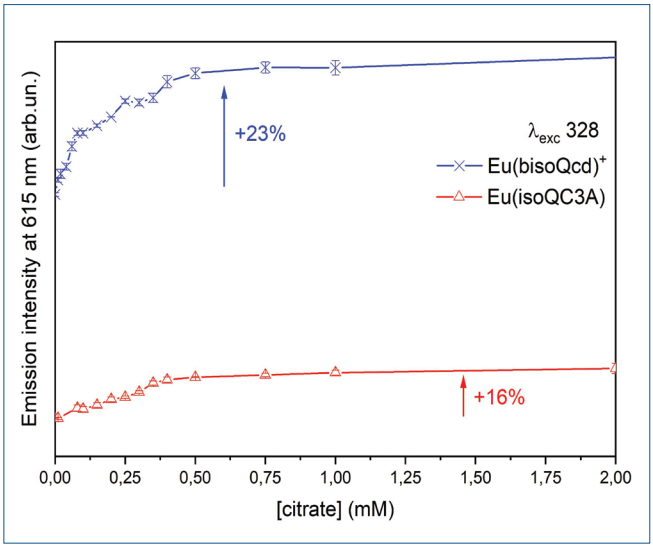


Fig. 6 - Evolution of the Eu(III) emission intensity upon titration (in simulated extracellular fluid) of Eu(*biso*Qcd) and Eu(*iso*QC3A) with citrate. The matrix contains BSA (0.4 mM), $HCO_3^-$ (28 mM), $Na_2HPO_4$ (1.3 mM), L-lactate (2.3 mM) and $Na_2SO_4$ (0.6 mM). The concentration of the complexes was 80 μM

## Luminescence determination of citrate in complex matrix

Despite the similar affinity of both Eu(III) complexes towards the three analytes ($HCO_3^-$, BSA and citrate), one could expect a higher sensitivity towards the analyte (citrate) which causes the largest increase of the quantum yield once it interacts with the complex. Driven by this conviction, four titration experiments were done, simulating a real interstitial extracellular fluid with respect to its main constituents. The evolution of the Eu(III) luminescence emission upon titration with each analyte in presence of the respective other two constituents (at their concentrations in extracellular fluid) is reported in Fig. 5. A good selectivity of the optical response towards citrate is observed in the case of the Eu(*biso*Qcd) complex. No significant changes of the luminescence intensity are recorded during the titration when BSA and $HCO_3^-$ are added (experiments 1 and 2). On the other hand, a significant increase of the luminescence (around 30%, experiment 3) is detected upon addition of citrate, up to its extracellular concentration. Similar conclusions can be drawn in the case of Eu(*iso*QC3A) (Fig. 5): the total increase of the luminescence due to the presence of citrate is around 25%. In another experiment (Fig. 6), an even more complex matrix was used, to further highlight the capabilities of our Eu(III) probes for citrate determination. Here, citrate was added to a matrix containing all the main analytes present in an extracellular fluid (L-lactate, sulfate, hydrogen phosphate, BSA and $HCO_3^-$ at their related concentrations). Also, in this case for both complexes, the luminescence intensity increases upon addition of citrate, 23% and 16% for Eu(*biso*Qcd) and Eu(*iso*QC3A), respectively (Fig. 6). It is obvious from Fig. 6 that citrate can be determined quantitatively with both complexes in complex matrices up to concentrations of 500 μM.

## Conclusions

The isoquinoline-based complexes Eu(*biso*Qcd) and Eu(*iso*QC3A) have proved to be excellent probes for the optical detection of citrate anions in complex matrix, such as interstitial extracellular fluid. They show good solubility and thermodynamic stability in water and a high selectivity of the optical response towards the citrate anion. In the case of Eu(*biso*Qcd) complex, a total increase of around 30% of the Eu(III) luminescence intensity at 615 nm was detected when the extracellular concentration of citrate is reached in a complex matrix containing $HCO_3^-$ and BSA, as main interfering analytes. This increase is still as high as at 23% when all major constituents of interstitial extracellular fluid are present ($HCO_3^-$, BSA, L-lactate, hydrogen phosphate and sulfate). Despite the similar affinities of both Eu(III) complexes towards $HCO_3^-$, BSA and citrate, the selective optical response towards the latter analyte can be explained by considering the highest Eu(III) luminescence quantum yield of the corresponding adduct. Further application can foresee the development of an analytical method to measure the citrate level in interstitial extracellular fluid both in normal condition (0.1-0.3 mM of citrate) and in samples where the citrate concentration is altered (above 0.3 mM).

## Acknowledgments

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**Complessi di Eu(III) luminescenti come sensori per le biomolecole**

In questo progetto si è seguito un approccio multidisciplinare per la progettazione e la caratterizzazione di nuovi complessi di europio(III) basati sulla struttura di base chirale costituita dal frammento 1,2-diamminocicloesano. Tali complessi sono stati applicati per la rilevazione di importanti bioanaliti presenti nel liquido extracellulare, quali bicarbonato, albumina e citrato.