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# Rapid determination of total hardness in water using fluorescent molecular aptamer beacon

T. Mairal Lerga<sup>a</sup>, Ciara K. O'Sullivan<sup>a,b,\*</sup>

<sup>a</sup> Nanobiotechnology & Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Spain

<sup>b</sup> Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys 23, 08010 Barcelona, Spain

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## ABSTRACT

A double-labelled synthetic oligonucleotide is used as a fluorescent molecular aptamer beacon for the reagentless determination of total hardness in tap and bottled waters. Modified thrombin binding aptamer (5'-NH-C3-GGTTGGTGTGGTTGG-C3-SH-3') carrying 6-carboxyfluorescein (FAM) and 7-amino-4-methyl-coumarin labels at 5' and 3', respectively, was used for the simultaneous combined measurement of Mg<sup>2+</sup> and Ca<sup>2+</sup> cations. Interference from the K<sup>+</sup> cation is eliminated via selective tuning of the assay conditions, increasing the temperature beyond the melting point of the potassium-stabilised quadruplex facilitating its liberation from the quadruplex, whilst maintaining the integrity of the magnesium/calcium-stabilised structure. No interference from other cations found in tap or bottled water was observed. The detection limit of the aptamer beacon is 0.04 mmol L<sup>-1</sup>, with a dynamic linear range of 0–0.5 μM and is very reproducible, with an R.S.D. = 8%, n = 3. The fluorescent molecular beacon is applied to the determination of total hardness in tap and bottled waters and its' performance compared to that of the standard method of complexometric titration and atomic absorption spectroscopy, with an excellent correlation observed. Further work is focused on the immobilization of the aptamer for the development of a re-usable fluorescent/electrochemical aptasensor, for the determination of water hardness.

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## 1. Introduction

Determination of hardness in water, whether potable or domestic is of critical importance. Hard water causes scaling, which is the precipitation of minerals to form a deposit called lime scale. Scale can clog pipes and can decrease the life of toilet flushing units, as well as reducing detergent efficiency and thermal efficiency of air conditioners. In industry water hardness contributes to scaling in boilers, cooling towers and other industrial equipment and water hardness is monitored constantly to avoid costly breakdowns. Additionally, these ions,

along with alkaline ions, are the most important factor in cardiovascular diseases and can potentially affect the health of both humans and animals [1], and according to a range of studies, from a health point of view, calcium and magnesium are 40–80 mg L<sup>-1</sup> [2] and 20–30 mg L<sup>-1</sup> [2,3], respectively, with a total water hardness of 2–4 mmol L<sup>-1</sup>. Water hardness is not defined in a unified manner, multiple units have been used to express it [4] and it refers to the total concentration of alkaline earth ions in water [5–9]. However, as the concentrations of Ca<sup>2+</sup> and Mg<sup>2+</sup> are usually much greater than all other ions, it has generally been accepted that hardness can

\* Corresponding author at: Nanobiotechnology & Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Spain. Tel.: +34 977 558740/8722; fax: +34 977 669621/8205.

E-mail addresses: [ciara.osullivan@urv.net](mailto:ciara.osullivan@urv.net), [ckosulli@etse.urv.es](mailto:ckosulli@etse.urv.es) (C.K. O'Sullivan).

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be equated to the sum of calcium and magnesium concentrations, determined by EDTA titrimetric method and expressed in  $\text{mmol L}^{-1}$  (ISO, 1984)<sup>1</sup> or as a  $\text{CaCO}_3$  equivalent in  $\text{mg L}^{-1}$  (standard methods, 1998).<sup>2</sup> The standard method for the determination of water hardness is complexometric titration, but is affected by several metal ions, is time-consuming [5], and laborious although the flow-batch technique has managed to decrease assay time and reagent consumption [10]. Atomic absorption spectroscopy (AAS) is also used for the determination of hardness in water but is lengthy and necessarily laboratory based technique. For trace metal analysis AAS has recently been replaced by inductively coupled plasma-optical emission spectrometry (ICP-OES) [11]. Although great efforts have been expanded to synthesize  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  selective ionophores to elaborate sensors [1,6,12], interferences as well as matrix effects and irreproducibility seriously affect the accuracy of the analysis.

Given the increasing need for automated, rapid water hardness analysis, many reports on continuous-flow type formats have appeared in the literature. A fibre optic sensor using fluorescent transduction and a polyether antibiotic has been reported for detection of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  demonstrating good detectability and selectivity, but suffering from drawbacks of a short lifetime and long assay time [13]. Continuous flow formats have been reported for ISEs [14–17], UV-vis spectrophotometry [18–22] and FASS [23]. Some rapid-test assays have been commercialised, such as the AQUADUR total hardness test sold by Macherey-Nagel [24], water hardness test strips from Serim research corporation [25] and the EM Quant total hardness test from Cole Parmer [26]. Numata et al. described a divalent cation selective electrode, with nearly equal selectivity to  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , and the electrode was applied to tap water and upland soil extracts and validated with standard method using chelatometric titration. Large interferences from heavy metal and transition metal ion are, however, observed, although interference from  $\text{Na}^+$  and  $\text{K}^+$  ions are very small [27]. Saurina et al. reported on the use of potentiometric sensor arrays for the determination of calcium and total hardness in neutral waters, and the authors present a very interesting, novel approach to improve selectivity of the calcium and magnesium ISEs, eliminating the effect of interference from other cation species, using chemometric data treatment for the multivariate sensor array demonstrating the possibilities of applying chemometrics to potentiometric sensors, but also highlighting the drawbacks of ISEs for determination of water hardness if this mathematical approach is not employed [6]. Capitan Valley et al. reported a new approach to determine water hardness using a non-continuous, reversible optical strip consisting of a polyester sheet as a support, with a transparent zone containing a K22B5 bipodal azocrown ether in poly vinyl chloride for combined detection of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  with a high discrimination over other cationic species. Cross-reactivity with other cations routinely found in tap and mineral waters was observed,

although the approach demonstrated four times more selectivity for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  than for  $\text{Na}^+$  and fifteen times more than for potassium [1]. Lima et al. have described a flow-batch-technique for a simple and rapid approach for a simple YES/NO response on water hardness control, with a high sample throughput of 120 samples for hour and is highly reliable with low consumption of chemicals. However, the method is not quantitative but does have an application in cut-off quality control.

It is well known that aptamers, synthetic DNA or RNA, constitute an alternative for analytical applications in therapeutics, diagnostics and bioanalytical applications; with advantages such as their smaller size, the possibility of labelling without affecting their affinity, integrity and folding conformation offering them great versatility. Furthermore, as they are artificial oligonucleotides, aptamers demonstrate a high resistance to temperature, pH or the presence of hazardous reagents. Regarding the folding ability of the aptamers, specifically those that contain single or multiple guanine-rich which segments are known to form four-stranded helical conformations in solution, the conversion from random coil to G-quadruplex structure is strongly dependent on the presence of certain counterions.

One of the techniques used to explore the structural properties of G-quadruplexes is fluorescence spectroscopy, in particular Förster/fluorescence resonance energy transfer (FRET) [28]; this methodology offers the capability to study the conformational changes due to induction of the G-quadruplex following interaction with other molecules or ions. In FRET assays the biomolecule is labelled with two fluorescent markers, a donor and an acceptor; if the distance between the two fluorophores is optimal the efficiency is measured by monitoring the decrease in fluorescence emission of the donor or the increase in fluorescence emission of the acceptor. There are several reports described in the literature that exploit this technology for detection of ions using G-quadruplex structures [29–31].

Taking advantage of this particular characteristic of guanine rich aptamers, several reports have been appeared where G-quadruplex oligonucleotides have been used for the fluorescent detection of specific cations, such as  $\text{K}^+$  [29–34],  $\text{Tb}^{3+}$  [35] and  $\text{Pb}^{2+}$  [36,37]. In our laboratory we have used the thrombin-binding aptamer (TBA) for the selective detection of  $\text{K}^+$  in the presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (removed using chelators) and in this work we report the selective detection of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the presence of  $\text{K}^+$ , exploiting the thermal stability of the  $\text{Ca}^{2+}/\text{Mg}^{2+}$  stabilized quadruplex structure, and the application of the fluorescent beacon to the determination of total hardness in tap and bottle water (Fig. 1).

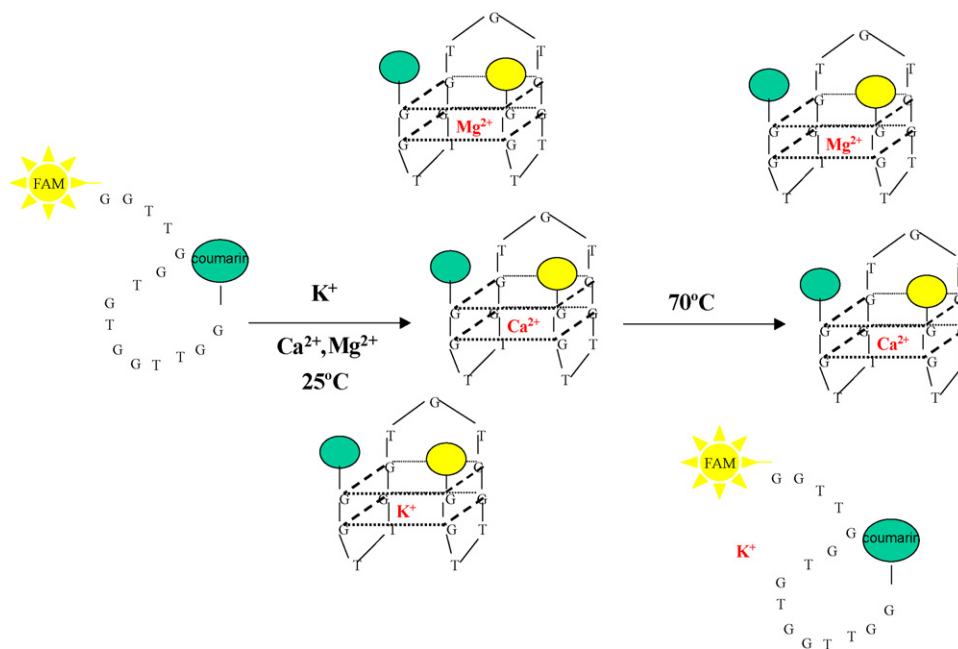
## 2. Experimental

### 2.1. Materials

A variant of the thrombin-binding aptamer (TBA, 5'-NH-C3-GGTTGGTGTGGTTGG-C3-SH-3') was provided by Molecular Solutions (London, UK). Dyes for the synthesis of fluorescent molecular beacon, carboxyfluorescein succinimidyl ester (6-FAM) and 7-amino-4-methyl-coumarin, were purchased from

<sup>1</sup> ISO 6059: 1984 water quality – determination of the sum of calcium and magnesium – EDTA titrimetric method.

<sup>2</sup> ASTM D 511-93 (1998) standard test methods for calcium and magnesium in water.



**Fig. 1 – Schematic of approach used to selectively detect magnesium and calcium in the presence of potassium for determination of total water hardness.**

Invitrogen (Barcelona, Spain) and Sigma (Barcelona, Spain) respectively, sulfo-SMCC was provided by Cultek (Barcelona, Spain). Deionised water was produced with a Milli-Q RG system (Millipore Ibérica, Madrid, Spain). Tap water was obtained from various sites in Tarragona, Spain and the bottled mineral waters analyzed were Gourmet, Viladrau, Manantial de St. Aniol, Solan de Cabras and Fontvella.

#### 2.1.1. Buffer preparation

Phosphate buffer (10 mM, pH 7.4) was prepared from its corresponding phosphate salts,  $NaH_2PO_4$  and  $Na_2HPO_4$ , to a concentration of 10 mM and then NaCl was dissolved up to a concentration of 50 mM.

Ammonia buffer solution was prepared using 7% of  $NH_4OH$  and 1% of  $NH_4Cl$  1% and adjusting the pH to 10.

HEPES buffer was prepared from 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 mM adjusting the pH to 8.4.

#### 2.2. Equipment

The spectrophotometric measurements were performed with a spectrophotometer, Cary Eclipse from Varian with Cary temperature controller. The atomic absorption spectrophotometric measurements were performed with an atomic absorption spectrophotometer with background correction by Zeeman effect Hitachi Z8200. The fluorescent assays were performed with a fluorescence spectrophotometer, Cary Eclipse from Varian with Cary temperature controller.

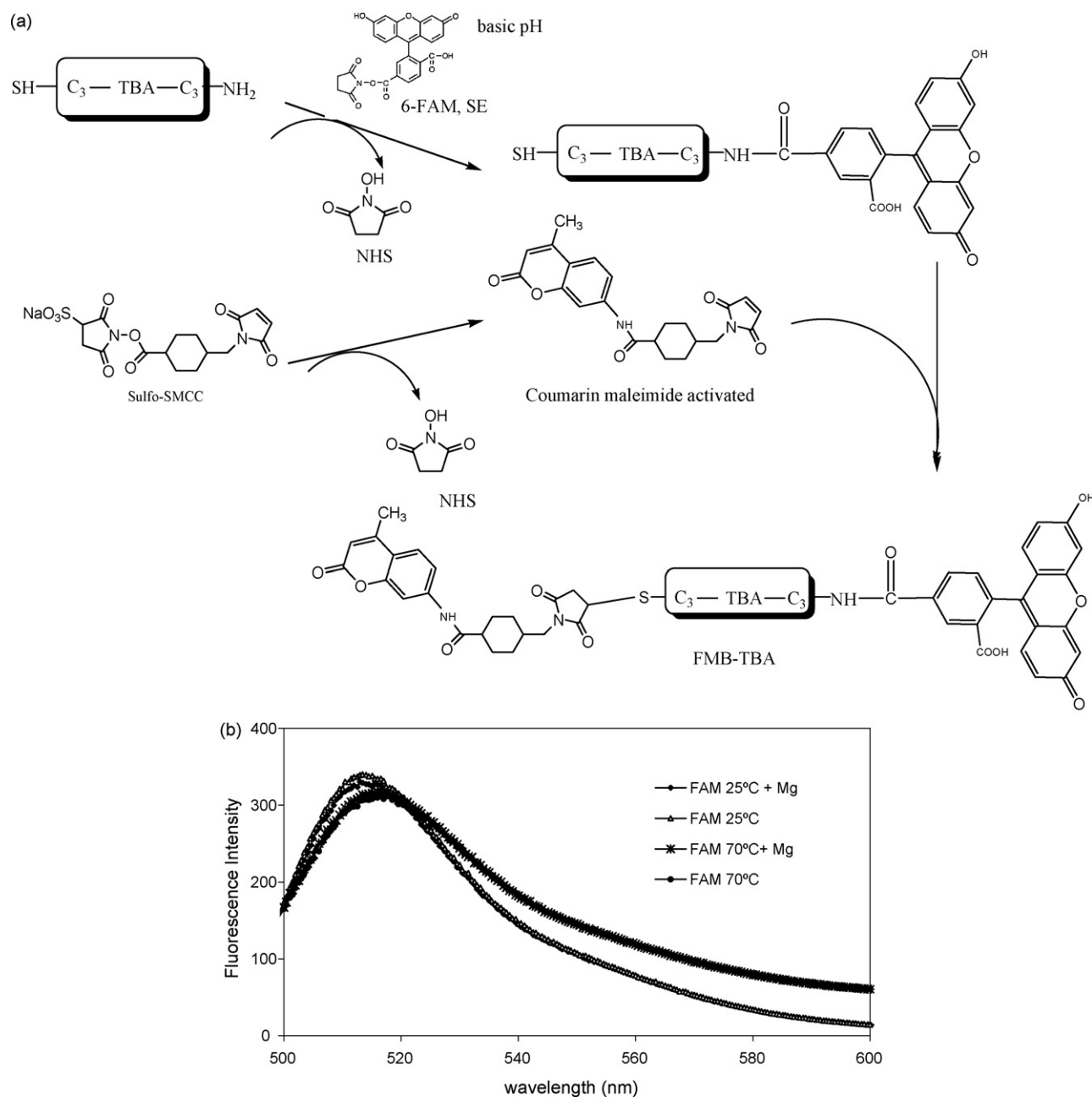
#### 2.3. Synthesis of fluorescent molecular beacon

The fluorescent molecular beacon was produced by incorporation of a 6-FAM fluorophore label to the 5'-ter of the

NH-C3-TBA-C3-SH and coumarin label to the 3'-ter of the modified aptamer (Fig. 2). Briefly, 22 nmols of DNA (100  $\mu g$ ) were incubated for 4 h in 100  $\mu L$  100 mM sodium tetraborate solution containing 250  $\mu g$  of FAM (5 mM) solved in dimethylsulphoxide (DMSO). Subsequently, the conjugate was precipitated using ethanol, dried and coumarin (280 mM) dissolved in DMSO was introduced, using sulfo-SMCC (46 mM) in 10 mM PBS pH 7.4 as a coupling reagent. The mixture was incubated for 2 h in the dark at  $37^\circ C$ , followed by ethanol precipitation. The fluorescent molecular beacon was desalted using a G-25 column (Amersham-Pharmacia; Barcelona, Spain) and its concentration was evaluated using UV-vis spectrophotometry.

#### 2.4. Atomic absorption spectroscopy for the determination of levels of cations found in tap waters

The levels of various cations such as  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Mn^{2+}$ ,  $K^+$ ,  $Pb^{2+}$ ,  $Li^+$  and  $Fe^{3+}$ ,  $Cd^{2+}$  in tap water were determined using atomic absorption spectroscopy. Calibration curves were prepared using standard preparations of the cations studied and the samples were diluted with de-ionised water containing 1% (v/v)  $HNO_3$ , except in the case of  $K^+$  and  $Li^+$ , where 10% (v/v) lanthanum was added as an additional cation releasing agent. Each measurement was carried out in triplicate. For the preparation of the standards, stock solutions (1000 ppm) were used for each ion and dilution series with de-ionised water containing 1% (v/v)  $HNO_3$  and 10% (v/v) lanthanum in the case of potassium and lithium were carried out. The concentration ranges in  $mg L^{-1}$  used for the counterions studied were as follow:  $Cu^{2+}$  [0, 1, 2, 3, 4, 5, 8];  $Mg^{2+}$  [0, 0.1, 0.2, 0.3, 0.4];  $Mn^{2+}$  [0, 0.5, 1, 1.5, 2];  $Zn^{2+}$  [0, 0.1, 0.2, 0.4, 0.6];  $Ca^{2+}$  [0, 0.4, 0.8, 1, 1.2, 1.6, 2];  $K^+$  [0, 1, 2, 3, 4, 10];  $Li^+$  [0, 1, 2, 3, 4, 10];  $Pb^{2+}$  [0, 1, 2, 4, 8];  $Cd^{2+}$  [0, 0.1, 0.2, 0.3, 0.4];  $Fe^{3+}$  [0, 1, 2, 3, 4].



**Fig. 2 – (a) Schematic synthesis procedure of thrombin-binding aptamer fluorescent molecular beacon (FMB-TBA), 6-FAM and coumarin were covalently introduced to the thiolated amino modified TBA sequence. (b) Scan determination of 6-carboxy-fluorescein (FAM),  $\lambda_{\text{ex.}} = 490 \text{ nm}$ . The experiment was done at 25 °C and 70 °C with and without magnesium.**

### 2.5. Effect of counterions on fluorescence quenching of molecular aptamer beacon

The effect of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{K}^{+}$  cations were studied using the fluorescent molecular beacon (TBA-FMB). The assay was carried out using 1  $\mu\text{L}$  of the fluorescent molecular beacon stock solution (100  $\mu\text{M}$ ), diluted to 100 nM in HEPES (10 mM, pH 8.4), and the solution was placed in a quartz cell with 1 cm path length. A dilution series of each cation (final concentration 0.5–5 mM) was added to the MB solution and fluorescent emission was measured ( $\lambda_{\text{ex.}} = 494 \text{ nm}$ ;  $\lambda_{\text{em.}} = 518 \text{ nm}$ ) before and after addition of each.

### 2.6. Temperature ramping experiments

The effect of the temperature on the TBA-FMB conformation in the presence of  $\text{K}^{+}$  (100 mM),  $\text{Mg}^{2+}$  (100 mM),  $\text{Ca}^{2+}$  (100 mM),  $\text{Cu}^{+}$  (0.1 mM),  $\text{Zn}^{2+}$  (0.015 mM),  $\text{Fe}^{3+}$  (50  $\mu\text{M}$ ) and  $\text{Pb}^{2+}$  (1 mM) was studied measuring the fluorescence in a temperature gradient, where the temperature was ramped from 25 °C to 75 °C, at a rate of 1 °C/min<sup>-1</sup>. The fluorescent molecular beacon stock solution (100  $\mu\text{M}$ ) was diluted to 100 nM in HEPES 10 mM pH 8.2. The effect of temperature on FAM was also measured in the presence of each counterion as a control. Calibration curves were prepared for  $\text{K}^{+}$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  at both 25 °C and 70 °C.

## 2.7. Analysis of real tap and bottled water samples

### 2.7.1. ISO standard method of compleximetric titration

The determination of total hardness of water by compleximetric titration was carried out as previously reported [38]. For each sample the titration was carried out in triplicate.

### 2.7.2. Fluorescent molecular aptamer beacon assay

For the determination of total hardness of water using the fluorescent molecular beacon, a calibration curve was prepared using calcium and magnesium standards in the range of 0–3000  $\mu\text{M}$  (2  $\mu\text{L}$ ) were added to 148  $\mu\text{L}$  of 100 nM of TBA-FMB diluted in HEPES 10 mM pH 8.4.

The fluorescent emission of the beacon was measured ( $\lambda_{\text{ex.}} = 494 \text{ nm}$ ;  $\lambda_{\text{em.}} = 518 \text{ nm}$ ) before and after addition of each standard. The assay was carried out at 70 °C and the concentration of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in  $\mu\text{M}$  was plotted against the percentage of quenching. (Fig. 5) A linear regression of quenching (%) = 9.4273 + 0.016816 [ $\mu\text{M Ca}^+/\text{Mg}^{2+}$ ],  $r^2 = 0.998$ , was obtained. The % quenching can be defined as

$$\text{quenching (\%)} = \frac{F_0 - F_x}{F_0} \times 100$$

where  $F_0$  is the fluorescence prior to sample addition and  $F_x$  is the fluorescence following sample addition.

Bottled and tap water samples were diluted in HEPES 10 mM pH 8.4 to fall within the dynamic linear range and the diluted water sample was added to a 150  $\mu\text{L}$  of the TBA-FMB solution (100 nM). All bottled samples were diluted 1:10 and tap water samples 1:8. Fluorescence was measured after and before each addition. The assay was carried out at 70 °C.

## 3. Results and discussions

### 3.1. Atomic absorption spectroscopy for the determination of levels of cations found in tap waters

As it is known that several cations induce the TBA to undergo a conformational change from random coil to the quadruplex structure, a study was carried out to determine the concentration of these and other cations known to be present in tap and bottled water. As can be seen in Table 1, the most abundant cation in tap water is calcium ranging from 98 to 100  $\text{mgL}^{-1}$ , followed by magnesium, between 35 and 26  $\text{mgL}^{-1}$ , potassium with lower values from 15 to 4  $\text{mgL}^{-1}$ , around 1  $\text{mgL}^{-1}$  of zinc, and in some rare cases copper and iron. The other counterions assayed, such as  $\text{Mn}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Pb}^{2+}$  and  $\text{Cd}^{2+}$  were not detectable

**Table 1 – Atomic absorption assay of different tap water samples**

Sample	Ion ( $\text{mgL}^{-1}$ )									
	Ca	Mg	Zn	Cu	Mn	Li	K	Pb	Cd	Fe
TW1	98	33	0.39	ND	ND	ND	4.65	ND	ND	ND
TW2	81	35	0.92	0.99	ND	ND	4.35	ND	ND	ND
TW3	380	34	0.25	ND	ND	ND	4.40	ND	ND	0.37
TW4	89	26	0.25	ND	ND	ND	15	ND	ND	ND
TW5	100	26	ND	ND	ND	ND	14.9	ND	ND	ND

by atomic absorption spectroscopy, so their presence was irrelevant for this study.

### 3.2. Spectral properties of the fluorescent molecular beacon

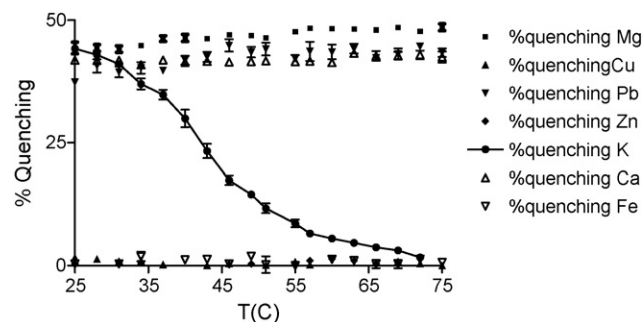
In order to demonstrate the quenching effect induced in the FMB-TBA in the presence of certain counterions, sample solutions of the FMB-TBA with and without magnesium were excited at different wavelengths. Excitation of the fluorescent molecular beacon at  $\lambda = 490 \text{ nm}$  produces an emission peak at  $\lambda = 518 \text{ nm}$ , corresponding to the emission of 6-FAM. As it was expected, when magnesium is present in the sample there is a decrease of the emission peak of 6-FAM due to the quenching process. This quenching effect was observed at both 25 °C and 70 °C, demonstrating that the use of FAM-TBA-coumarin conformational change as a fluorescence detection system following the spectral changes of FAM ( $\lambda_{\text{ex.}} = 490$ ;  $\lambda_{\text{em.}} = 518 \text{ nm}$ ).

A control was carried out to ensure that magnesium does not produce any change on the 6-carboxyfluorescein spectral properties, scan determination of FAM was done in the presence and absence of magnesium at both temperature 25 °C and 70 °C (Fig. 2b).

### 3.3. Temperature effect on the TBA-FMB in presence of certain counterions

The ions found to be present in tap water samples were then tested for their potential interference with the TBA-FMB, either by having an effect on the fluorophore, FAM, or by inducing quadruplex formation. Another abundant ion present in bottle and waters that have been softened by ion exchange methods is sodium. The effect of this counterion on the TBA-FMB was already studied [39] concluding that the addition of a high wide range of this monovalent metal had no effect on the beacon fluorescence.

The effect of the counterions studied was analysed ramping the temperature from 25 °C to 75 °C and it was concluded that FAM is not affected by any of the cations studied (data not shown). In Fig. 3 it can be seen that  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{K}^+$  are the only cations that stabilize quadruplex formation resulting in concomitant quenching of fluorescence. Copper, zinc and iron did not produce an effect on the TBA-FMB conformation in all range of temperatures at the ion concentration studied.



**Fig. 3 – Effect of the temperature on the TBA-FMB (100 nM) in presence of different salts KCl (100 mM), ZnCl<sub>2</sub> (0.015 mM), Cu (0.1 mM), MgCl<sub>2</sub> (100 mM), CaCl<sub>2</sub> (100 mM), FeCl<sub>3</sub> (50  $\mu\text{M}$ ) and PbCl<sub>2</sub> (1 mM).**

The constant ion concentration was determined in accordance with the results obtained by atomic absorption spectroscopy which were coherent with that bottle and tap water studies reported in Refs. [9,11] and permitted by the European Union Drinking Water directive [40].

As potassium is present in tap water at quite high concentrations (up to  $15 \text{ mg L}^{-1}$ ), in order to determine total hardness of water, it was necessary to eliminate the potassium interference, and temperature ramping experiments were carried out to see if the differentiation between  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  could be achieved via temperature tuning. The melting curves for the Mg/Ca/K stabilized G-quartet shown in Fig. 3, clearly indicate that the Mg/Ca complexes are extremely stable at high temperatures, whereas the  $T_m$  of the  $\text{K}^+$  stabilized quadruplex is  $\cong 45^\circ\text{C}$ , in agreement with previously obtained results used DSC to calculate the  $T_m$  on the TBA in the presence of 100 mM of potassium [41]. This differentiation in thermal stability of the TBA in the presence of  $\text{Ca}^{2+}/\text{Mg}^{2+}/\text{K}^+$  allows selective and specific detection of water hardness via the combined detection of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ .

### 3.4. Ion concentration effect on G-quadruplex stability of TBA-FMB

The enhanced stability of the  $\text{Mg}^{2+}$ , induced quadruplex formation is mentioned in a recent publication [42], where the authors postulate the enhanced effect due to  $\text{Mg}^{2+}$  is due to the formation of a quadruplex–dimer structure. Thus, by choosing a temperature notably higher than  $45^\circ\text{C}$ , the quenching of fluorescence would be exclusively due to  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and not  $\text{K}^+$  as is represented in Fig. 4. The wide ion concentration range used in this study was in agreement with data obtained using reference methodologies but it should be note that concentrations of higher than 100 mM and lower concentrations down to a nanomolar scale were tested and the same behaviour was observed in all the concentration ranges studied. The high sensitivity observed is particularly important for the application of the reported fluorescent beacon approach to chemically treaded industrial process waters, such as boiler feed waters where very low detection of water hardness is required (Fig. 5).

### 3.5. Analysis of real tap and bottled water samples

Calibration curves for  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{K}^+$  were constructed carrying out the assay at  $25^\circ\text{C}$  and  $70^\circ\text{C}$  and as can be observed

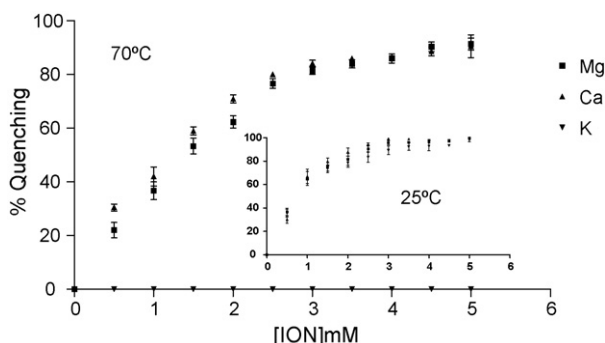


Fig. 4 – Effect of counterions on TBA-FMB. The assay was carried out at  $25^\circ\text{C}$  and  $70^\circ\text{C}$ .

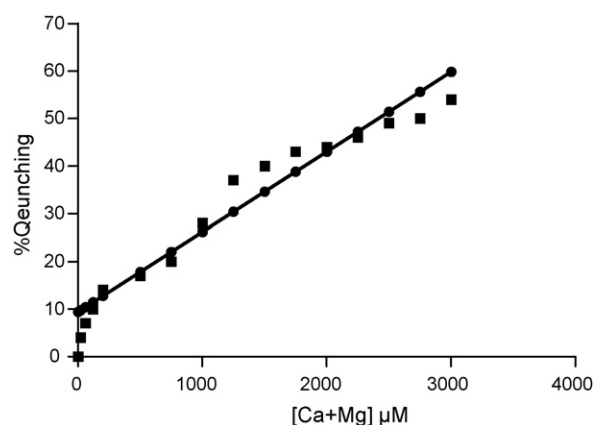


Fig. 5 – Calibration curve obtained plotting the concentration of calcium and magnesium against the percentage of quenching at  $70^\circ\text{C}$ .

Table 2 – Determination of hardness of tap and bottled water samples using fluorescent molecular beacon and reference method

Sample	Ref. (mM $\text{CaCO}_3$ )	Molecular beacon (mM $\text{CaCO}_3$ )	%Recovery
TW1	1.2	1.3	97
TW2	1.0	1.5	90
TW3	4.2	4.3	99
TW4	1.2	1.4	92
TW5	1.3	1.4	97
BW1	0.17	0.13	96
BW2	0.24	0.21	96
BW3	0.12	0.10	99
BW4	0.6	0.89	75
BW5	0.9	0.12	91

For tap water samples, the reference method used was atomic absorption spectrometry, and for the bottled mineral water samples, the EDTA-eriochrome T complexometric method was used.

the potassium ion is completely liberated from the TBA at  $70^\circ\text{C}$  and these conditions can thus be used for interferent-free, reagentless and rapid determination of total water hardness. As the calibration curves for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are identical, either one can be used for the determination of the sum of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (i.e. total hardness) in a water sample. As water hardness has home and health implications, a range of tap waters and bottled waters were tested using the molecular beacon approach as well as the standard method of complexometric determination and an excellent correlation obtained (Table 2).

## 4. Conclusions

We report here a fluorescent molecular beacon for the detection of total hardness in tap and bottled waters. The molecular beacon provides a reagentless, reproducible, sensitive, easy to use assay, with the entire assay being completed within 30 s, with a cost of 0.05€ per assay.

The assay is very reproducible and has been applied to real tap and bottled water samples, and the results obtained compared to that of the standard methods of atomic absorption

spectrometry and complexometric titrations, and an excellent correlation obtained. The molecular beacon can be easily formatted for use in situ, as to high-throughput flow-through systems, and current work is focused on immobilisation of the aptamer for the development of a re-usable aptasensor for continuous flow-through detection of total water hardness.

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