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Measurements of calcium with a fluorescent probe Rhod-5N: Influence of high ionic strength and pH

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Abstract

We describe a new method for the spectroscopic determination of high calcium concentration using a fluorescent probe Rhod-5N. This method was investigated in order to be utilized in high ionic strength solution, such as seawater. The probe is fluorescent when bound to calcium, LM, but not as the free form L. The dissociation constant of the equilibrium (0.14 mM) was determined at several ionic strengths, i.e. in the absence and in the presence of additional ions (0.7 M NaCl). The influence of pH was studied. In order to correctly model the experimental data, we included a new fluorescent compound: LHM (calcium bound protonated probe). The first acidity constant (0.02 μM) and the second dissociation constant (4.5 mM) were calculated. A useful range for the determination of calcium concentration is provided. Such a method is fast and easy to carry out.

Keywords: Rhod-5N; Fluorescence; Probe; Calcium; Data analysis; Seawater

1. Introduction

Formation and dissolution of calcium carbonate in the ocean are important players in the global carbon cycle and are intimately related to the control of atmospheric CO_2. This gas, one of the most abundant green house gases in the earth's atmosphere, is thought to be mostly absorbed by the oceans and ultimately neutralized by the reaction with $CaCO_3$ in marine sediments. The precipitation and dissolution of calcium carbonate are a function of $[Ca^{2+}]$ and $[CO_3{}^{2-}]$ concentrations as well as the calcite and argonite solubility constants. In the open ocean, variations of Ca^{2+} concentrations are rather small and related with salinity variations. However, a direct determination of $[Ca^{2+}]$ can help in the determination of $CaCO_3$ saturation state and in the understanding of the carbon cycle.

In 1976, Lebel and Poisson [1] proposed a potentiometric titration of magnesium and calcium ions in seawater. The method uses the differences between ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol bis-(β-aminoethyl ether)-*N*-*N*′ tetraacetic acid (EGTA) dissociation constants. They obtain, in ideal conditions, a reproducibility of 1/1000. However, this method is long and demanding. It requires stable environment to avoid erroneous measurements and it cannot be adapted for on board measurements, as a result, it is barely used. Recently, a number of teams attempted to develop new methods using electrophoresis [2], plasma atomic spectrometry [3,4] and near-infrared spectroscopy [5]. We make use of our knowledge of fluorescent probe to conceive new calcium measurements. Molecular probe, Inc. provides calcium probe with high dissociation constant, the highest belonging to Rhod-5N. Fluorescent measurements are fast and require small volume of seawater (<1 ml). Nowadays, companies can provide small spectrofluorimeter that can be easily brought on boat.

In this paper, we described the probe interaction with calcium and proton. We check the influence of high ionic strength on the measurement. We propose a methodology and a model to determine calcium quantity in high ionic strength solution, such as seawater. Finally, we test the reproducibility of the method and give a useful range of optimal utilization.

2. Experimental

2.1. Chemicals

$CaCl_2$, NaCl, $HgCl_2$ and EDTA are of analytical grade and are used as received. A buffer solution of Titrisol at pH 8 is

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used. No calcium concentration higher than residual calcium is detected in this solution. Addition of 10% of the buffer, give a stable pH (8.05 ± 0.02). Rhod-5N was purchased from Molecular Probe, Inc. Aliquots of 40 μl Rhod-5N at 0.6 mM are kept at −20 °C. Before use, the solution is diluted to the final volume of 1 ml with de-ionized water. The final concentration is tested by recording a fluorescence spectrum for each new solution. Ultrapure de-ionized water was generated by a MilliQ plus system (Millipore).

2.2. Fluorescence measurements

Emission fluorescence spectra are recorded with a spectrofluorimeter (Flx, SAFAS, Monte Carlo, Monaco) after excitation at 551 nm. At this wavelength and at the working concentration, the fluorescence intensity of the probe is more than thousand times higher than the combined intensities of the Raman band and the dissolved organic material in seawater samples. Residual calcium is found in the water used for the experiments. It has to be regularly checked that the residual concentration does not exceed 10 μM. The glassware is washed with concentrated solution of acid (HCl) and rinsed with de-ionized water. If required, EDTA solution is used.

Three different experiments were performed for K_d determination, for pH variation and for reproducibility of Ca^{2+} determination.

2.2.1. K_d determination

When the fluorescent spectra of the free probe L and the bound probe LM are different, K_d can be determined studying the evolution of the LM fluorescence spectra in presence of M. Knowing K_d the relation can be used to calculate the concentration of M. For these experiments, the concentration of probe L is chosen constant and we vary the concentration $[M]_{total}$ of calcium. Two solutions are prepared: solution A containing Rhod-5N (0.3–0.6 μM), buffer at pH 8 (10%) and residual calcium, and solution B, containing specific calcium concentration in solution A. Varying concentrations of calcium are prepared by mixing different amounts of the two solutions A and B. If necessary, the pH is adjusted with micro-drop of NaOH. The fluorescence spectra are recorded for up to 14 calcium concentrations between 10 μM and 20 mM.

2.2.2. pH variation

With calcium chelating dyes, protonated and non-protonated probes show two different fluorescent forms [6]. We study the evolution of the LM fluorescent spectra in presence of H^+ proton. This was done in order to determine at which pH the non-protonated form exists alone. For these experiments, microvolumes of NaOH or HCl are added to fresh solution B directly in the glass spectrocell. pH is measured with a pH-meter (PHN 81, Tacussel) with an accuracy of 0.01. The fluorescent spectra are recorded for 20 solutions ranging from 4 to 10.

2.2.3. Reproducibility

For Ca^{2+} determination experiments, the reproducibility of the results is checked preparing several solutions of the same calcium concentrations with various NaCl concentrations. In Table 1, 20 μl of the probe (24 μM), 10% of buffer (pH 8) and 100 μl of NaCl (0.7 M) are mixed directly in the glass spectrocell with 100 μl of calcium (10 mM). De-ionized water is added to obtain 1 ml final volume. After spectrum recording, 25 μl of calcium (4 M) are added to the solution. This is done in order to check the variation of the total probe concentration $[L]_{total}$. The pH is increased with micro-drops of NaOH to a value between 8.5 and 10. The probe spectrum in the solution with an excess of calcium is then recorded. The temperature is regulated at 25 ± 0.1 °C.

Table 1
Mean and standard deviation (S.D.) of Rhod-5N fluorescence intensities and calculated calcium concentrations for five identically prepared solutions

	Corrected[a] I_{LM} (a.u.)	Corrected[b] I_{max} (a.u.)	(Ca)[c] (M)	[Ca][d] (M)
	7461	22699	4.10×10^{-4}	1.00×10^{-3}
	7414	22086	4.23×10^{-4}	1.04×10^{-3}
	7697	22738	4.29×10^{-4}	1.05×10^{-3}
	7471	22746	4.10×10^{-4}	1.00×10^{-3}
	7662	22777	4.25×10^{-4}	1.04×10^{-3}
Mean	7442	22600	4.19×10^{-4}	1.03×10^{-3}
S.D.	180	290	0.08×10^{-4}	0.02×10^{-3}

[a] Correction factor for LH: 1.090 (Section 3.3.3).
[b] After addition of 25 μl Ca (4 M) to the 1 ml solution. Correction factors: dilution factor (1.025/1) and probe saturation factor: 1.060 (Section 3.2.2).
[c] [NaCl] = 0.07 M, $[L]_{total}$ = 0.48 μM and K_{dLM} = 1.4 mM.
[d] γ_{Ca} = 0.409.

2.3. Data analysis

Since LM is the only fluorescent form, LM concentration can be calculated from the experimental data obtained at constant volume:

$$[LM] = \frac{I_{LM}}{I_{max}} \times [L]_{total} \quad (1)$$

where $[L]_{total}$ is the total probe concentration and [LM] is the calcium bound probe concentration. I_{LM} is the intensity of LM at the equilibrium and I_{max} is the intensity if the entire probe is bound to calcium. It was initially determined after addition of an excess of calcium. After analysis of the fluorescence spectra, we obtain an experimental curve of LM concentration as a function of the total calcium concentration (Fig. 1) or as a function of pH (Fig. 2). The total calcium concentration is calculated considering the calcium concentration of solution B and calcium residual in solution A.

2.4. Theoretical model

We created a theoretical model in order to model the experimental data. A system of equilibrium equations defines the binding model. Basic hypotheses about individual species involved in these interactions are made as follows.