Thermodynamics and fluorescence emission studies on potential molecular chemosensors for ATP recognition in aqueous solution †

M.[a] Teresa Albelda,[a] M. Alexandra Bernardo,[b] Enrique Garcia-España,[c] M.[a] Luz Godino-Salido,[d] Santiago V. Luis,[e] Maria João Melo,[b] Fernando Pina[b] and Conxa Soriano[a]

[a] *Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Valencia, 46100 Burjassot (Valencia), Spain*
[b] *Departamento de Química, Centro de Química Fina e Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal. E-mail: fjp@dq.fct.unl.pt*
[c] *Departamento de Química Inorgánica, Facultad de Química, Universidad de Valencia, C/ Dr. Moliner 50, 46100 Burjassot (Valencia), Spain. E-mail: enrique.garcia-es@uv.es*
[d] *Departamento de Química Inorgánica y Orgánica, Universidad de Jaén, 23071 Jaén, Spain*
[e] *Departamento de Química Orgánica y Inorgánica, Universidad Jaume I (Castellón), Spain*

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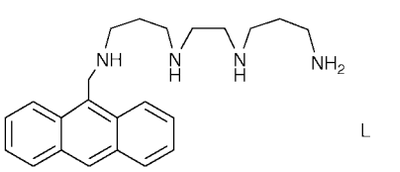
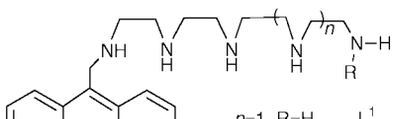
The interaction of the open-chain polyamine *N*-(3-aminopropyl)-*N′*-[3-(anthrylmethyl)aminopropyl]ethane-1,2-diamine (L) with the relevant anionic forms of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) is described. Unambiguous criteria for defining thermodynamic selectivity based on the use of effective stability constants are presented. The interaction of L and several other topologically similar polyammonium receptors with ATP has been shown to occur through electrostatic and π-stacking intermolecular forces. The π-stacking binding mode is modulated by the protonation degree of ATP as indicated by fluorescence emission titrations. Evidence for the use of these receptors as ATP luminescent chemosensors is advanced.

Introduction

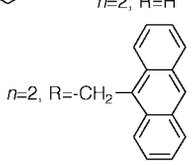
Nucleotide sensing and quantification by means of luminescent probes represent an important target in supramolecular chemistry due to their many biological and biomedical implications.[1] The first step required is the association of host and guest species to give a stable enough adduct. However, the magnitudes associated with anion recognition processes are not usually as large as those obtained for metal ions, particularly if water is the solvent of choice, since it competes in the stabilisation of the charged partners through a variety of intermolecular forces like hydrogen bonding. Therefore, very efficient sensors are required for the detection of these processes and, as a matter or fact, the literature concerning fluorescent chemosensors for anions is rather limited. Probably the most outstanding examples come from the work of Czarnick and Lehn's research groups.[2,3] The former author provided examples for the recognition of different carboxylate, sulfate and phosphate anions by polyammonium receptors based on the tripod polyamine tris(2-aminoethyl)amine (tren) attached by one of its primary amino groups to an anthracene signalling component. Lehn, Hosseini and Fenniri have recently prepared two multisite binding receptors based on the well-known macrocyclic structure of 1,13-dioxa-4,7,10,16,19,22-hexaazatetracosane (bisdien) to which one or two acridine luminescent groups, respectively, have been covalently bonded to the central nitrogen atoms of both polyamine fragments through aminopropyl side arms. The second receptor seemed to be particularly well suited for nucleotide recognition since, apart from displaying large variations in its emission properties upon coordination to nucleotides, it does not induce hydrolytic ATP cleavage. Other kinds of receptors developed by Lehn and Zinic based on bis(phenanthridinium) fragments displayed large quenching effects on their emission due to π-stacking interactions with the adenine moiety of the nucleotides.[4] These interactions gave constants as high as *ca.* 5.8 logarithmic units that were independent of the polyphosphate chain of the nucleotides.



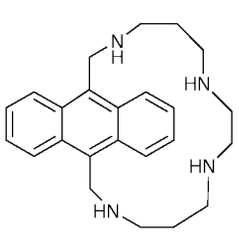
L



n=1, R=H L[1]
n=2, R=H L[2]



L[3]



L[4]

† Plots of the fluorescence emission intensity *versus* the molar ratio [ATP]/[L] for the systems ATP–L[1], ATP–L[2] and ATP–L[3] are available as supplementary data from BLDSC (SUPPL. NO. 57639, pp. 1) or the RSC Library. See Instructions for Authors available *via* the RSC web page (http://www.rsc.org/authors).

Herein we present some results of the interaction with nucleotides of some simple receptors (L–L^4) built up by assembling together a polyamine chain and an anthracene fragment. In spite of their apparent simplicity these receptors strongly interact with nucleotides achieving high degrees of selectivity for ATP. On the other hand, the presence of the anthracene unit permits the easy transformation of the chemical events into light signals.[5,6]

Experimental

Materials

Synthesis of receptors L and L^1 has been accomplished as described in ref. 7 following a modification of that reported in ref. 8. For obtaining L–L^2 an excess of the corresponding polyamines was reacted with anthracene-9-carbaldehyde in ethanol to give the corresponding imines. Reduction to obtain the free-amine was carried out with $NaBH_4$. L^3 was obtained by reacting in ethanol pentaethylenehexaamine with anthracene-9-carbaldehyde in a 1 : 2 molar ratio. Reduction of the diimine was carried out with $NaBH_4$. A detailed description of the synthetic procedure will be published elsewhere. L^4 was prepared as described in ref. 9. All compounds were purified and handled as their hydrochloride salts and characterised by elemental microanalysis and spectroscopy.

NaCl, used as background electrolyte, was a Merck suprapur product. CO_2-free NaOH solutions were prepared as described in ref. 10. The sodium salts of ATP, ADP and AMP were from Fluka with purity greater than 98%.

Spectrophotometric and spectrofluorimetric titrations

Absorption spectra were recorded on a Perkin-Elmer Lambda 6 spectrophotometer and fluorescence emission on a SPEX F111 Fluorolog spectrofluorimeter. $HClO_4$ and NaOH were used to adjust the pH values that were measured on a Metrohm 713 pH meter. The linearity of the fluorescence emission with concentration was checked in the concentration range used (10^{-5} mol dm^{-3}–10^{-6} mol dm^{-3}). The absorbance of the excitation wavelength was maintained lower than 0.15. When excitation was carried out at wavelengths different from the isobestic points, a correction for the absorbed light was performed. Fitting of the emission intensity *vs.* [substrate]/[receptor] curves was performed as described in ref. 4.

Emf measurements

The potentiometric titrations were carried out at 298.1 ± 0.1 K in NaCl 0.15 mol dm^{-3}. The experimental procedure used (burette, potentiometer, cell, stirrer, microcomputer, *etc.*) was the same as has been fully described elsewhere.[10] The acquisition of the emf data was performed with the computer program PASAT.[11] The reference electrode was an Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen-ion concentration probe by titration of previously standardised amounts of HCl with CO_2-free NaOH solutions and determining the equivalent point by the Gran's method,[12] which gives the standard potential, $E°$, and the ionic product of water ($pK_w = 13.73(1)$).

The computer program HYPERQUAD,[13] was used to calculate the protonation and stability constants. The titration curves for each system (*ca.* 100 experimental points corresponding to at least three measurements. The pH range investigated was 2.5–10.5 and the concentration of the different anions and of L ranged from 1×10^{-3} to 5×10^{-3} mol dm^{-3}). Protonation constants for L were taken from ref. 7. Protonation constants of ATP, ADP and AMP are: ATP, log $K_{HA/A\cdot H} = 6.38$ (1), log $K_{H_2A/HA\cdot H} = 3.96(1)$ and log $K_{H_3A/H_2A\cdot H}$ *ca.* 1.7: ADP, log $K_{HA/A\cdot H} = 6.16(1)$ and log $K_{H_2A/HA\cdot H} = 3.96(1)$; AMP, log $K_{HA/A\cdot H} = 6.06$ (1), log $K_{H_2A/HA\cdot H} = 3.96(1)$.

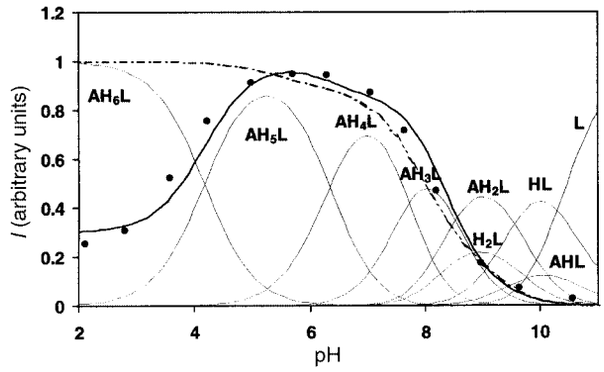


Fig. 1 pH dependence of the fluorescence emission intensity of the compound L: in the absence of ATP (----); in the presence of 3-fold ATP excess (●); molar fraction distribution of the adducts (—); molar distribution of the free receptor in the presence of ATP (----). Fitting was achieved by a linear combination of the several molar fractions of all the species involved using the same coefficients as in the case of L in the absence of ATP, except for H_6AL^{4+} in which the coefficient was reduced from 1 to 0.3 indicating the existence of a quenching process.

The different titration curves for each system were treated either as a single set or as separate curves without significant variations in the values of the stability constants. Finally, the sets of data were merged together and treated simultaneously to give the final stability constants. Moreover, several measurements were made both in formation and in dissociation (from acid to alkaline pH and *vice versa*) to check the reversibility of the reactions.

Results and discussion

In Table 1 are presented the cumulative formation constants for the interaction of L with the nucleotides ATP, ADP and AMP determined in 0.15 mol dm^{-3} NaCl at 298.1 ± 0.1 K. Fig. 1 shows, as an example, the distribution diagrams for the systems ATP–L.

Several features regarding speciation and thermodynamic aspects of these interactions deserve discussion. First of all, the stoichiometries found for the adducts formed in all systems are always 1 : 1 as inferred from the analysis of the titration curves and from the variations of the ^{31}P chemical shifts with the molar receptor–substrate ratio for given pH values. The protonation degrees of the adducts detected vary from 1 to 6 for ATP, from 2 to 6 for ADP and from 2 to 5 for AMP. The distribution diagrams show that, particularly in the case of ATP, the adduct species clearly predominate in a wide pH range (see Fig. 1).

Since the different receptors and the nucleotide themselves participate in several protonation equilibria that are often overlapped, care has to be taken in order to decide which are the right stepwise equilibria representative of the formation of the adduct species. For instance, in the case of ATP taking into account the protonation constants of ATP and of L,[7] the species HAL^{3-}, H_2AL^{2-} and H_3AL^{-} should involve the interaction of the fully deprotonated ATP (A^{4-}) with the corresponding protonated forms of the receptor.

$$A^{4-} + H_jL^{j+} = H_jAL^{(j-4)+}\ j = 1,2,3 \qquad (1)$$

The same reasoning allows us to infer that H_6AL^{2+} is formed by interaction of the fully protonated form of the receptor H_4L^{4+} and the diprotonated ATP (H_2A^{2-}). The remaining species, H_5AL^{+} and H_4AL, are formed in a pH region where overlapping occurs and it is not so easily established which are the right species contributing to their formation. For instance, H_5AL^{+} could be formed either through the equilibria (2) or (3),

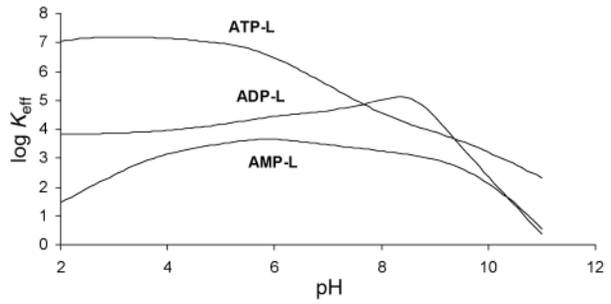


Fig. 2 Plot of the effective conditional constants *vs.* pH for the systems ATP–L, ADP–L and AMP–L.

$$H_3L^{3+} + H_2A^{2+} = H_5AL^{+} \quad (2)$$

$$H_4L^{4+} + HA^{+} = H_5AL^{+} \quad (3)$$

with equilibrium constants 8.7 and 7.1, respectively, or from both of them with a partial participation which will depend on pH.

At first sight, it is difficult to anticipate which of these equilibria will best describe the adduct formation and thus which will be the proper magnitudes of the stability constants. This analysis can be extended to all the systems presented in this paper and to all those systems in which receptors and substrates are involved in overlapped protonation equilibria.

This obvious point has often given rise to misinterpretations of the constants and thus of the parameter of thermodynamic selectivity. To overcome these difficulties some of us proposed a method based on calculating the distribution diagrams for the ternary system substrate A–substrate B–receptor, and representing the overall percentages of free and complexed substrates as a function of pH.[14] This method had the advantage of not requiring any assumption on the location of the protons in the host and guest species and allowed for establishing proper selectivity ratios.

Here we present a straightforward way to define selectivity ratios based on the use of effective stability constants. This method also affords valuable hints for establishing the right protonation degrees of the intervening species. For a given pH value, if the total amounts of free substrate ($\Sigma[H_iA]$), free receptor ($\Sigma[H_jL]$) and adduct formed ($\Sigma[H_{i+j}AL]$) are known one can define an effective stability constant (eqn. (4)).

$$K_{eff} = \Sigma[H_{i+j}AL]/\Sigma[H_iA]\,\Sigma[H_jL] \quad (4)$$

In Fig. 2 are presented plots of the logarithms of the effective stability constants as a function of pH for all three systems ATP–L, ADP–L and AMP–L. In this Figure it can be seen that the ATP effective constants are clearly higher than those of the ADP and AMP adducts over a wide pH range (pH 2–9). The quotient of the effective constants at a given pH allow for establishing selectivity ratios and for instance at pH 5 the selectivity ratios ATP/ADP and ADP/AMP would be *ca.* 600 and 5, respectively. This method would allow comparison between any systems provided the measurements have been carried out under the same experimental conditions.

On the other hand, as the effective stability constants for the ATP–L system are in the range 2.3–7.2 logarithmic units, all the ways of expressing equilibria with values of the constants far outside this range can be discarded. In this respect equilibrium (2) can not be that responsible for the formation of the species H_5AL^{+}, indeed in the pH range 3–6 where this species is the main one in solution, the logarithms of the effective stability constants take values no higher than 7.2 (see Fig. 2), confirming that HA^{3-} and H_4L^{4+} are the main species involved in the formation of the penta-protonated adduct.

Table 1 Cumulative and representative stability constants for the systems ATP–L, ADP–L and AMP–L determined at 298.1 K in 0.15 mol dm^{-3} NaCl

Reaction	A = AMP^{2-}	A = ADP^{3-}	A = ATP^{4-}
$A + 2H + L \rightleftharpoons H_2A$ [a]	22.64(3) [b]	22.36(5)	23.47(3)
$A + 3H + L \rightleftharpoons H_3A$	30.61(2)	30.63(4)	31.99(3)
$A + 4H + L \rightleftharpoons H_4A$	37.14(2)	37.84(3)	39.71(3)
$A + 5H + L \rightleftharpoons H_5A$	42.19(2)	42.94(4)	46.05(3)
$A + 6H + L \rightleftharpoons H_6A$	—	46.64(7)	50.22(4)
$A + HL \rightleftharpoons HAL$	—		3.1
$A + H_2L \rightleftharpoons H_2AL$	3.1	2.9	4.0
$A + H_3L \rightleftharpoons H_3AL$	3.5	3.5	4.8
$HA + H_2L \rightleftharpoons H_3AL$	5.1	5.0	6.3
$A + H_4L \rightleftharpoons H_4AL$	4.4	5.1	7.0
$HA + H_3L \rightleftharpoons H_4AL$	3.9	4.5	6.3
$HA + H_4L \rightleftharpoons H_5AL$	3.4	4.0	7.1
$H_2A + H_4L \rightleftharpoons H_6AL$		3.8	7.2

[a] Charges omitted for clarity. [b] Values in parentheses are standard deviations for the last significant figure.

Table 2 Cumulative and representative stability constants for the systems ATP–L^1, ATP–L^2 and ATP–L^3 (where ATP = A) determined at 298.1 K in 0.15 mol dm^{-3} NaCl

Reaction	L^1	L^2	L^3
$A + H + L \rightleftharpoons HAL$ [a]	14.22(3) [b]	12.9(1)	13.00(2)
$A + 2H + L \rightleftharpoons H_2A$	23.70(3)	22.74(3)	21.88(2)
$A + 3H + L \rightleftharpoons H_3A$	32.24(2)	31.76(3)	30.10(2)
$A + 4H + L \rightleftharpoons H_4A$	39.32(2)	39.88(2)	37.50(2)
$A + 5H + L \rightleftharpoons H_5A$	45.22(2)	46.65(2)	44.07(2)
$A + 6H + L \rightleftharpoons H_6A$	49.49(2)	51.98(3)	49.30(3)
$A + 7H + L \rightleftharpoons H_7A$	52.50(2)	55.80(3)	53.21(3)
$A + 8H + L \rightleftharpoons H_8A$	—	58.00(5)	55.95(3)
$A + 9H + L \rightleftharpoons H_9A$	—		58.97(9)
$A + HL \rightleftharpoons HAL$	3.95	2.92	3.97
$A + H_2L \rightleftharpoons H_2AL$	4.27	3.51	4.63
$A + H_3L \rightleftharpoons H_3AL$	4.98	4.11	5.26
$HA + H_2L \rightleftharpoons H_3AL$	6.43	6.15	6.47
$A + H_4L \rightleftharpoons H_4AL$	7.1	5.5	6.59
$HA + H_3L \rightleftharpoons H_4AL$	5.68	5.85	6.28
$A + H_5L \rightleftharpoons H_5AL$	9.9	8.1	9.09
$HA + H_4L \rightleftharpoons H_5AL$	6.62	5.93	6.78
$H_2A + H_3L \rightleftharpoons H_5AL$	7.7	8.7	8.97
$HA + H_5L \rightleftharpoons H_6AL$	7.79	7.04	7.94
$H_2A + H_4L \rightleftharpoons H_6AL$	7.01	7.38	8.13
$HA + H_6L \rightleftharpoons H_7AL$	—	8.53	—
$H_2A + H_5L \rightleftharpoons H_7AL$	6.92	6.98	7.97

[a] Charges omitted for clarity. [b] Values in parentheses are standard deviations for the last significant figure.

This analysis extended to the three systems studied has allowed us to select the equilibria presented at the bottom of Table 1 as the correct ones for describing the formation of all adducts. As can be seen, in several cases two equilibria are required for defining the formation of the adducts. For instance, for the formation of H_4LA^{+} ($A = ATP^{4-}$) equilibria (5) and (6) have to be considered. Obviously the extent of participation of each one of them will change throughout the pH range in which the adducts exist in solution.

$$HA^{-} + H_3L^{3+} = H_4LA^{2+}, \log K = 6.3 \quad (5)$$

$$A^{2-} + H_4L^{4+} = H_4LA^{2+}, \log K = 7.0 \quad (6)$$

The interaction of ATP has also been studied for receptors L^1, L^2 and L^3 containing larger chains and in the case of L^3 two anthracene fragments. The constants of the representative equilibria for these systems are included in Table 2. It is interesting to note the similar affinities that all receptors display for ATP in spite of the different lengths of the chain (see the plot of the effective constants in Fig. 3).

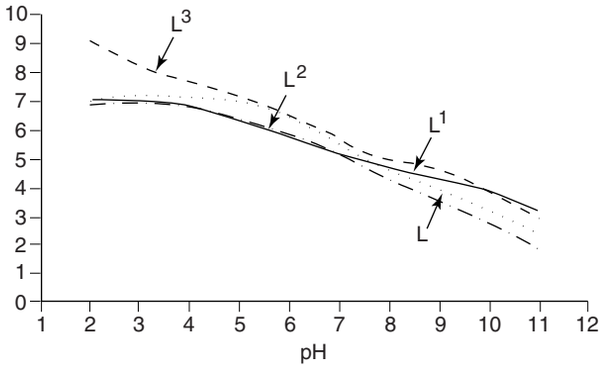


Fig. 3 Plot of the effective conditional constants *vs.* pH for the systems ATP–L, ATP–L^1, ATP–L^2 and ATP–L^3.

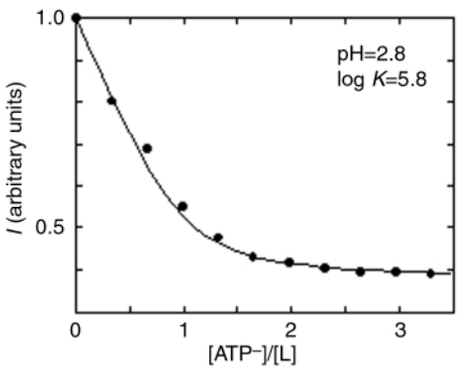


Fig. 4 Fluorescence emission intensity *vs.* [ATP]/[L] at pH = 2.8. Fitting was achieved for log K = 5.8.

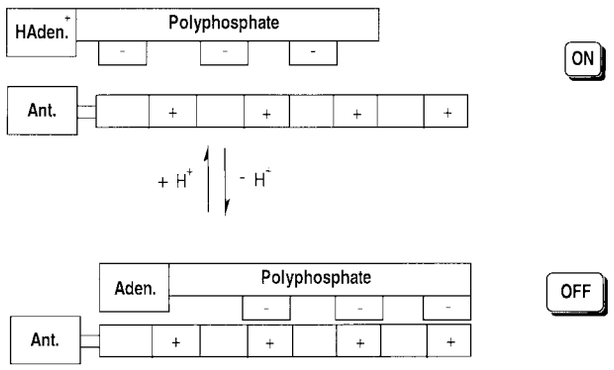
Fluorescence emission studies

Fluorimetric titrations for the system L–ATP show a bell-shaped curve in which light-emission is strongly quenched for pH values lower than 4, then emission is recovered up to pH 7 to drop down again and definitely disappears above pH 10. This behaviour may be interpreted on the basis of the different species formed in solution and in the light of the luminescent behaviour of the free receptor.[7]

Spectrofluorimetric titrations of the free-receptor allowed us to infer that H_4L^{4+} and H_3L^{3+} were the only emissive species with relative fluorescence emission quantum yields $\varphi_{(H_4L^{4+})}/\varphi_{(H_3L^{3+})}$ of 0.85. In the presence of ATP, these species continue emitting independent of the fact that they are either complexing ATP or free, except for the H_6AL^{2+} species formed by the association of the fully protonated receptor L and the di-protonated form of ATP, which gives rise to a quenching effect. Comparing the fluorescence emission titration curves of the free and complexed receptor (Fig. 1, dotted and continuous lines respectively), the small increase in the chelation enhanced fluorescence (CHEF) emission observed in the ATP–L system at neutral pH can be attributed to the increase in apparent basicity of the receptor occurring upon co-ordination to ATP. Perhaps the most striking feature in this system is the dramatic chelation enhanced quenching (CHEQ) effect (almost 60%) observed below pH 4 (see Fig. 1). A plausible interpretation for such quenching could be a π-stacking interaction between the anthracene unit of the receptor and the adenine fragment of the guest species induced by the protonation of N3 of the adenine ring.[15] Evaluation of the stability constant at pH 2.0 provides a value of 5.8 logarithmic units (Fig. 1). Interestingly enough, the interaction of the receptor with the monoprotonated form of ATP does not yield such a quenching effect. Probably, a different matching between the triphosphate chain of $HATP^{3-}$ and the polyammonium fragment of L alters the relative disposition of the aromatic moieties in both molecules preventing an efficient π-stacking from occuring (see Scheme 1). When checking the behaviour of ADP, AMP, adenine and tripolyphosphate it was observed that only the first one produced CHEQ but with a much lower constant, log K = 3.7 at pH = 2.2.

Table 3 Truth table of the logic operation NAND

[H^+]	[ATP]	Emission (419 nm)
0	0	1
0	1	1
1	0	1
1	1	0



Scheme 1

Therefore, it seems that the molecular topology of the receptor is a key point in the facilitation of the π-stacking pattern. Indeed, experiments carried out with the anthracenophane 2,6,9,13-tetraaza[14](9,10)anthracenophane (L^4) do not show any significant effect. On the other hand, the analogous ligands containing the same fluorophore but different polyamine chains (L^1,L^2) provide similar CHEQ effects with very close association constants (L^1, log K_s = 5.6; L^2, log K_s = 5.4, both at pH 2.2, Supplementary Material). It is to be remarked that the constants obtained are not affected by the length and number of nitrogen atoms in the chain but only by the molecular arrangement of the receptor. The values of these stability constants are, on the other hand, also similar to those obtained by Lehn and Zinic for the interaction of ATP and other nucleotides with their bis(phenantridinium) based receptors.[4] However, it has to be noted that in our case, such stability constants are only obtained for ATP and not for other nucleotides or adenine itself.

A related receptor can be built appending two methyl anthracene fragments at both ends of a linear pentamethylene hexaamine chain (L^3). Interaction of L^3 with ATP also promotes a very significant quenching of the emission at low pH values allowing for calculating an association constant of log K = 5.7 at pH = 2.2 (Supplementary Material).

The similarity of the constants obtained, at acidic pH, for the ATP binding with all receptors, together with the lack of significant effects for AMP and adenine suggest that achieving important CHEQ effects requires as above discussed: i) a certain degree of anchorage of the polyphosphate chain of ATP by the polyammonium fragments of the receptor and ii) topological complementarity of the partners.

Logic gates

Switches based on systems operating in liquid solution have been described recently. De Silva and co-workers[16] reported several examples of these systems constituted of a receptor and a fluorophore separated by a spacer. The input signal is obtained through the binding of metal ions or protons to the receptor, while the output signal is generally detected by the

presence/absence of light emission. In a different approach, based on a pseudo rotaxane, Balzani and co-workers[17] were able to conceptualize an XOR logic gate. Following these ideas, the system ATP–L represents an example of a NAND logic gate as shown in Table 3. The initial state would be constituted of the free receptor in the pH range $5 < pH < 8$. The inputs are obtained by: i) addition of protons in order to reach a final pH of *ca.* 2, and ii) addition of a slight excess of ATP at pH 6. We consider a final output of 1 when the intensity of the fluorescence emission goes over 0.8 relative units, and of 0 when the intensity of the emission is below 0.4 relative units (see Fig. 1).

In the absence of any input the system shows fluorescence emission and thus the output signal is 1. The same output is obtained if only ATP or protons are added to the solution. When both inputs are present then a quenching effect occurs (Fig. 1) and the output becomes 0. This behaviour constitutes in terms of the Boolean logic a NAND response. The most interesting feature of the present systems is the fact that they can operate with anions apart from metal ions[7] and therefore, open new possibilities in the design of molecular logic devices.

These results demonstrate the potential use of these simple receptors as efficient luminescent ATP chemosensors or molecular devices. Further work is currently being performed in order to obtain related receptors with improved binding characteristics.

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