SYNTHESIS AND INVESTIGATION OF FLUORESCENCE SENSOR AND LABEL MOLECULES

Ph.D. Thesis Summary

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Budapest, 2011.

1. Introduction

This PhD thesis summarizes the results of our research conducted in two separate fields of science that are connected by the fluorescence phenomenon. The first part consist of our mechanistic studies on photoinduced electron transfer (PET) sensors. We found an anomalous behaviour in the signal transfer process, which could not be interpreted by the well-accepted working mechanism of these sensors. Inspired by these observations, we studied the possibility of constructing a new mechanism model, which could offer explanation for signal evolution in cases where the classic theory fails. The second part of the thesis is about the synthesis of biologically applicable fluorescent labels that are furnished with a so-called bioorthogonal function (azide or alkyne). These fluorescent dyes that show excellent photostability and possess remarkable Stokes shifts were found useful reagents e.g. in cell membrane labeling experiments using Copper Catalyzed Azide-Alkyne 1,3-diploar Cycloaddition (CuAAC) reaction. In spite of the popularity of the area little effort has been made to develop bioorthogonal labels in this spectral regime.

2. New scientific results

For the aformentioned reasons, the discussion of our results is divided into to main sections.

2.1. Photoinduced electron transfer (PET) sensors

A large number of today's fluorescent chemosensory systems exploit the photoinduced electron transfer (PET) phenomenon. Guest binding to the receptor unit changes the redox potential of an adjacent donor site, which reverses its ability to quench the fluorescence of the fluorophore part resulting in measurable fluorescence enhancement (OFF–ON systems). The generality and efficiency of this switching process led to the common use of PET sensors in numerous fields from simple ion sensing to logic gates. The effect of guest binding on the PET process is mostly rationalized by the guest's coordinative interaction with the donor site. There are certain cases for common PET sensors, however, where the formation of secondary interactions between the guest and the donor site in the host does not give a satisfactory explanation for the observed change of fluorescence. This suggests that in these sensors change in the conformational dynamics of the receptor part might also play a significant role in signal generation, which has been studied for some unique structures. The role of steric perturbation and conformational constraints at binding site has rarely been investigated in detail so far.

The starting point of our research was the observation that the mobility of an electron-rich module of the sensor has great influence on the signalling potential. This research suggested the existence of a new model, which is based on conformational dynamics. In an earlier report the research group where I started to conduct my research introduced a new sensor design, which exploited the influence of conformational dynamics on the PET process.

As a continuing effort at understanding signal generation in PET sensors, we aimed at tracking down the effects of conformational dynamics on the signal generationc process in more common sensory systems.

I. Four 18-crown-6 based sensors were selected for our studies. Compound **1** contains an azacrown host unit and an attached coumarin fluorophore, while **2a–c** possess a 1,10-diazacrown core with either two coumarin units (**2a**) or pendant coumaryl and benzyl (**2b**) or *tert*-butoxycarbonyl-methyl groups (**2c**) (Figure 1).

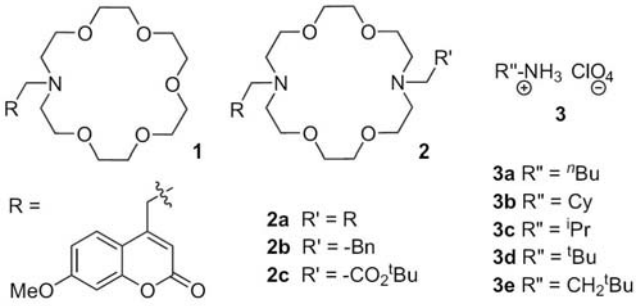

Figure 1. *Novel PET sensors*

II. We have examined the strength of the complexation between selected guests and sensors in a series of ^{1}H NMR spectroscopic experiments. The stoichiometry of the complexes between *n*-butylammonium perchlorate (**3a**) and **1** or **2a** was established as 1:1 by continuous variation method (Job's plot) (Figure 2).

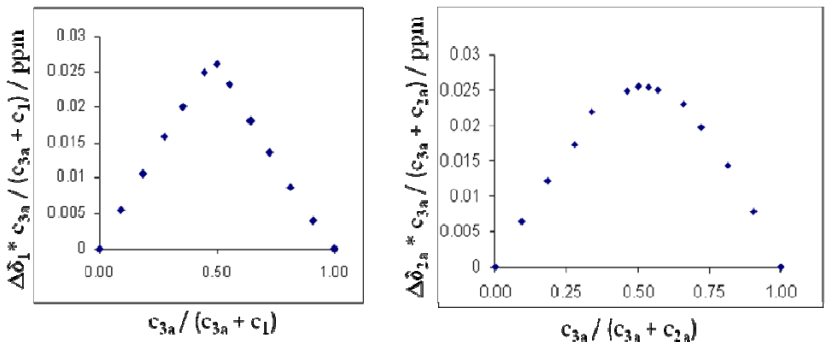

Figure 2. *Job's plot for* (**1-3a);** (**2a-3a)** complexes

We also determined the stability constants for selected sensor (**1** and **2a,b**)–guest (**3a,d,e**) complexes. NMR titrations showed that, of the studied sensors, **1** has the highest affinity toward the ammonium salts, and **2a** the lowest. In the case of **2b**, the determined stability constants lie between those of **1** and **2a**, although for ammonium salts of increased steric demand the experimental values are closer to **1**.

III. To establish the efficiency of the PET process in the studied sensors (that also corresponds to the highest available signal on complexation), the fluorescence enhancements (FEs) observed on their protonation using excess HBF_4 were examined. We compared the fluorescence of the sensor molecules and their protonated forms, where the PET process is completely blocked. Sensors bearing a diazacrown moiety have all shown increased signaling potential, compared to **1**, regardless of the number of the fluorophore units. The fluorescence enhancements in the **2** series were 3–4 times higher than that of **1**. The lower signaling ability of **1** originates in the less efficient fluorescence quenching by PET in its non-protonated form (compared to **2a–c**). Since we have ruled out the existence of *syn*-alignment of the fluorophores in the structure of **2a** by fluorescence and X-ray studies, we accounted the conformational mobility of the donor site's surroundings for fluorescence quenching. We presume that restricting the conformational freedom of the crown moiety statistically increases the occurrence of such conformers, where the donor site is more efficient in quenching the fluorescence.

IV. We also conducted a series of coordination experiments with different organic ammonium salts including *n*-butyl, cyclohexyl, isopropyl, *tert*-butyl, and neopentylammonium perchlorates (**3a–e**, respectively). The ammonium salts were selected on the basis of their similar binding modes but various steric demand. The measured fluorescence enhancements (FEs) for the different host–guest combinations showed that in case of sensors **1** and **2a**, the measured FE values with guests **3a–e** have a similar pattern, the values in the **2a** series being significantly higher, which is quite an interesting results knowing that the binding constants for **1** are **2** orders of magnitude higher than that for **2a** (Figure 3). The similar pattern in the enhancement values observed for **1** and **2a** can be rationalized on the basis of the proton-donating abilities (acidity) of guests **3a–d**. In **1**, the guest can approach the macrocycle without encountering steric hindrance of the chromophore, therefore, conformational changes in this sensor are mainly induced by direct coordination of the guest to the host's electron donor N-atom. Accepting that in its preferred conformation the fluorophore units in **2a** are located on the opposite sides of the

azacrown unit, sterically hindering the approach of the guest from both sides, it is evident that conformational changes on guest binding have to be more significant than that for **1** since the movement of at least one of the coumarins is a prerequisite for guest capturing. The change in the conformational characteristics of the macrocycle can lead to a significant decrease in the efficiency of the PET process and a highly increased fluorescence signal. Superposition of this dynamic effect on the effect of the secondary interactions (that are present in both sensors) results in an increased FE (Figure 4). Further evidence of the different measures of guest induced structural alterations in **1** and **2a** is provided by comparing the changes in the ^{1}H NMR chemical shifts of selected nuclei. Evidently, the guests have a more profound effect on the crown frame in the case of **2a**, which is in agreement with the results of the fluorescence measurements.

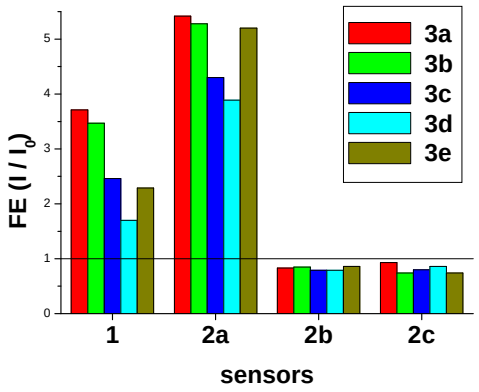

Figure 3. *FE values of sensors* **1**, **2a-c** *in the presence* **3a-e**

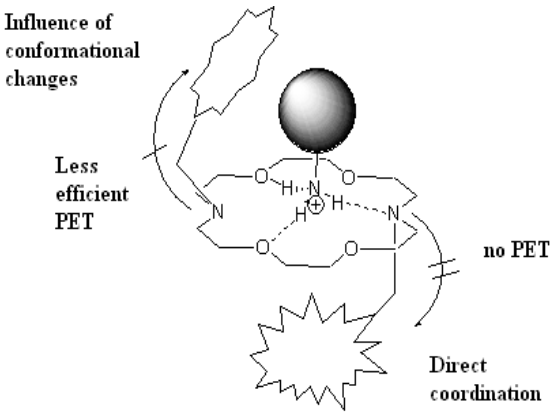

Figure 4. *Working mechanism of* **2a**

To extend our study, the complex formation between sensors **2b** and **2c** and ammonium salts **3a–e** was also examined in detail. Since the signaling potentials of **2b** and **2c** are similar to **2a**, we expected a similar complexation behavior. Interestingly, upon complexation of ammonium salts **3a–e** the fluorescence of **2b** and **2c** showed a moderate but persistent decrease. Consideration of the structural differences between **2b,c** and **2a** led to the assumption that in **2b** and **2c** the ring nitrogen atom next to the coumarin unit does not participate in the complexation. The reason behind this is that the benzylic- and the *tert*-butoxycarbonyl-methyl-amine moieties are significantly more basic than the coumarylmethyl-amine moiety. Assuming that guest binding in **2b** and **2c** occurs preferentially to the more basic nitrogen, this directs guest coordination opposite to the fluorophore's side, which leads only to the conformational changes in the macrocycle and no direct secondary interaction between the guest and the coumarylmethyl nitrogen. This means that the changed redox properties of the said nitrogen atom are the result of changes in the conformational dynamics of its environment. Such spatial arrangements might

become favorable around the coumarylmethyl nitrogen on guest binding that lead to a more efficient fluorescence quenching by PET. To provide experimental support for this, **2c** (where the various N-CH_2 signals could be differentiated) was titrated with acid and the process was monitored by ^{1}H NMR. On the addition of acid, the signal next to the ester unit showed a more significant change than the signal of the methylene group next to the coumarin unit. It is evident from this experiment that the protonation in this system occurs preferentially next to the tert-butoxycarbonyl-methyl moiety, supporting our assumption.

2.2. Synthesis of "clickable" fluorescent labels

In vitro and in vivo fluorescence imaging of biological structures that use near-infrared (NIR) fluorophores is becoming more and more important for their high sensitivity, excellent temporal and spatial resolution, and their potential for multichannel imaging. Fluorophores in the spectral region between the far-red and NIR region are particularly suitable for biological (both in vitro and in vivo) labeling as they are less or not at all interfered by biological background luminescence. Therefore there is an increasing demand for water-soluble labels fluorescing in the NIR regime. So far, little effort has been made to develop bioorthogonal labels in this spectral regime, so we wished to develop clickable NIR-dyes with large Stokes shifts. The work of Czerney *et al*. and our previous work on the development of the so-called "mega-Stokes" dyes has directed our attention to the polymethine scaffolds.

I. Using the synthetic scheme outlined in Figure 5 we have successfully synthesized two series of polymethine fluorophores bearing azide or alkyne functionalities.

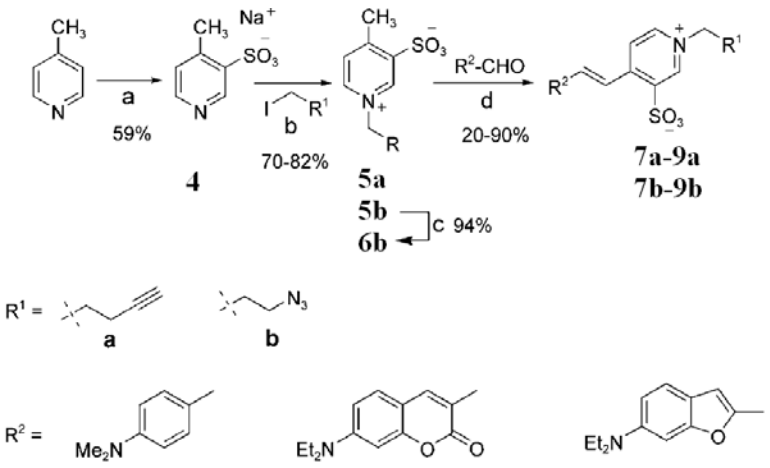

Figure 5. *Synthesis of clickable NIR fluorophores*

II. The fluorescence excitation and emission spectra of the dyes showed that each possesses a large Stokes shift (>100 nm). To our delight both excitation and emission spectra were shifted towards the NIR regime and emission maxima were positioned at 625, 674, and 734 nm for **7**, **8** and **9**, respectively (Table 1). The excitation spectra of the new dyes show that each of them is compatible with laser sources (e.g., Ar, He-Ne, diode lasers) widely used in fluorescence instrumentation such as cell sorters and imagers. The large Stokes shifts make these dyes ideal candidates for fluorescence resonance energy transfer (FRET) applications.

Dye	Solvent	λ_{max} (ex) [nm]	λ_{max} (em) [nm]	ε ($\times 10^4$) [$M^{-1}*cm^{-1}$]	Φ^a
7	MeOH	519	625	5,6	0,8
	PBS	523	630	4,3	-
8	MeOH	538	674 (695)	4,8	15,7
	PBS	544	675 (697)	5,3	1,0
9	MeOH	586	735	4,0	1,0
	PBS	588	744	2,8	-

Table 1. *Photophysical properties of clickable NIR fluorophores*
[a]:cresyl-violet was used standard

III. To test the feasibility of our clickable dyes in chemoselective tagging reactions the surface glycoprotein labeling of fixed cells supplied on azido sugar containing nutrients have frequently been used recently. We have adapted this system to demonstrate the ability of our dyes to undergo bioorthogonal labeling reactions efficiently. Prior to labeling, Chinese hamster ovary (CHO) cells were incubated with **7a–9a** to assess the possible cytostatic effects of these dyes. Based on experimental data, neither dye has a cytostatic effect on CHO cells. Subsequently, CHO cells were treated with azidoacetylmannosamine (ManNAz). The cells incorporated the azido sugar metabolically yielding azido sialic acid residues in their surface glycoproteins. The as-modified cells were then fixed then subsequently treated with **7a**, **8a**, or **9a**. The reaction was facilitated with $CuSO_4$-sodium-ascorbate and tris(benzyltriazolylmethyl)amine (TBTA) under physiological conditions. The results have shown that after 25 min reaction all labels had caused fluorescent tagging of the cells. We have also studied fluorescent labeling of azido-modified cells on the time scale. The fixed cells were incubated with **9a** for 5, 25, and 60 min. Results showed that even after 5 min reaction time efficient labeling could be observed (Figure 6).

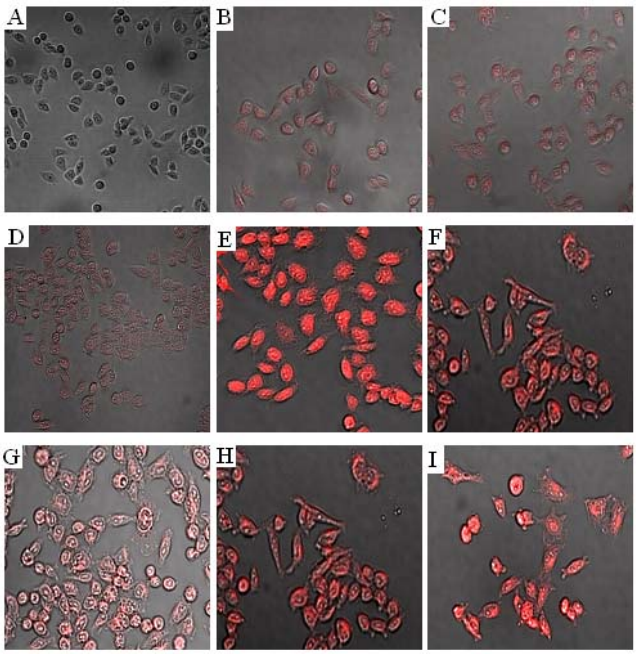

Figure 6. *Fluorescent microscopy images of CHO cells.*
Control experiments with cells not bearing azidosialic acid and treated with **7a**, **8a** and **9a** for 25 min (A, B, C); Cells modified with ManNAz and labeled with **7a**, **8a** and **9a** for 25 min (D, E, F); Time dependency: ManNAz treated cells labeled with **9a** for 5, 25 and 60 min (G, H, I).

3. Publications

Papers

Papers directly related to the thesis:

1. *Substituent dependent fluorescence response of diazacrown-based PET sensors*
Krisztina Nagy, Szabolcs Béni, Zoltán Szakács, Attila C. Bényei, Béla Noszál, Péter Kele and András Kotschy
Tetrahedron, **2008**, *64*, 6191-6195.

2. *Clickable long-wave "megaStokes" fluorophores for orthogonal chemoselective labeling of cells*
Krisztina Nagy, Erika Orbán, Szilvia Bősze, Péter Kele
Chemistry – An Asian Journal **2010**, *5*, 773-777.

Other papers:

3. *The development of conformational-dynamics based sensors*
Péter Kele, Krisztina Nagy, András Kotschy:
Angewandte Chemie, International Edition **2006**, *45*, 2565-2567.

4. *Clickable fluorophores for biological labeling – with or without copper*
Péter Kele, Xiaohua Li, Martin Link, Krisztina Nagy, András Herner, Krisztián Lőrincz, Szabolcs Béni, Otto S. Wolfbeis
Organic & Biomolecular Chemistry **2009**, *17*, 3486-3490.

Oral presentations:

1. Bioortogonális jelölésre alkalmazható polimetin alapú közeli IR fluorofórok szintézise és alkalmazása
Nagy Krisztina, Orbán Erika, Bősze Szilvia, Kele Péter
MTA Heterociklusos munkabizottsági ülés, Balatonszemes, 2009. május 20-22.

2. Bioortogonális jelölésre alkalmazható polimetin alapú fluorofórok szintézise és alkalmazása
Nagy Krisztina, Orbán Erika, Kele Péter:
IX. Clauder Ottó Emlékverseny, Budapest, 2009. április 23-24.

3. Szubsztituensfüggő fluoreszcencia-serkentés diazakorona alapú PET szenzorokban
Nagy Krisztina, Béni Szabolcs, Szakács Zoltán, Bényei Attila, Noszál Béla, Kotschy András, Kele Péter
MTA Heterociklusos munkabizottsági ülés, Balatonszemes, 2008.május 21-23.

4. Konformációs dinamikán alapuló koronaéter típusú szenzorok összehasonlító vizsgálata
Béni Szabolcs, Nagy Krisztina, Szakács Zoltán, Kele Péter
VIII. Clauder Ottó Emlékverseny, Budapest, 2007. április 12-13.

5. Development of conformational dynamics based sensors
Péter Kele, Krisztina Nagy, András Kotschy
1st European Chemistry Congress, Budapest, 2006, augusztus 27-31.

Poster presentations:

1. Polymetine based NIR click fluorophores for bioorthogonal labeling
Krisztina Nagy, Erika Orbán, Szilvia Bősze, Péter Kele
XI. Methods and Applications of Fluorescence, Budapest, 2009. szept. 6-9.

2. Fluorescent sensing effected by conformational mobility
Krisztina Nagy, Péter Kele, Szabolcs Béni, Zoltán Szakács, Béla Noszál, András Kotschy
X. Methods and Applications of Fluorescence, Salzburg, 2007. szept. 9-12.

3. Krisztina Nagy, Szabolcs Béni, Péter Kele, Zoltán Szakács, Béla Noszál, András Kotschy
Fluorescent sensing effected by conformational mobility,
XII. Blue Danube Symposium on Heterocyclic Chemistry, Tihany, 2007. június 10-13.

4. Nagy Krisztina, Béni Szabolcs, Kele Péter, Szakécs Zoltán, Noszál Béla, Kotschy András
Monoaza- és diazakoronaéter alapú szenzorok összehasonlitása
Centenáriumi Vegyészkonferencia, Sopron, 2007. máj.29-jún. 1.

5. Nagy Krisztina, Kele Péter, Bényei Attila, Béni Szabolcs, Szakács Zoltán, Noszál Béla, Kotschy András
Monoaza- és diazakoronaéter alapú szenzorok összehasonlítása
XII. Nemzetközi Vegyészkonferencia, Csíkszereda, 2006. október 3-8.
Diákposzter kategória, I.díj

6. Péter Kele, Krisztina Nagy, András Kotschy
Development of conformational dynamics based sensors
1st European Chemistry Congress, Budapest, 2006. aug. 27-31.