Bioanalytical Chemistry

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Indicator Displacement Assays Inside Live Cells**

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Abstract: *The macrocycle p-sulfonatocalix[4]arene (CX4) and the fluorescent dye lucigenin (LCG) form a stable host–guest complex, in which the dye fluorescence is quenched. Incubation of live V79 and CHO cells with the CX4/LCG chemosensing ensemble resulted in its spontaneous uptake. Subsequent addition of choline, acetylcholine, or protamine, which have a high affinity for CX4 and are capable of entering cells, resulted in a fluorescence switch-on response. This can be traced to the displacement of LCG from CX4 by the analytes. The results establish the principal functionality of indicator displacement assays with synthetic receptors for the detection of the uptake of bioorganic analytes by live cells.*

Indicator displacement assays (IDAs) with synthetic receptors have gained increasing attention in (bio)analytical chemistry[1] because they offer a supramolecular approach for the sensitive detection and differentiation of analytes. IDAs bypass both the need to construct highly specific antibodies for immunoassays and the design of nanotechnological sensing systems, for example those based on graphene oxide[2] or capped mesoporous[3] architectures. The measurement principle is based on the use of an indicator dye bound to a receptor (the "reporter pair"). The receptor, frequently a macrocyclic host, is chosen to have a high affinity for the target analyte, such that competitive binding leads to a release of the dye, which in turn results in a change of its photophysical properties, preferably its fluorescence. A characteristic of such assays is the low selectivity with which the receptor binds different analytes, which can be an advantage because a broad range of analytes can be targeted without the need to synthesize a specific receptor for each of them. On the other hand, it is a disadvantage because the assays are very sensitive to competitive binding by other species with complementary charge and size, which are omnipresent in many fluid matrices and are almost always present in biological samples.[4] Nevertheless, several biologically relevant applications of macrocycles have been brought forward, including the delivery of drugs,[5] improved membrane passage of fluorescent dyes[6] and DNA,[7] the immobilization of cells,[8] protein recognition,[9] and cell imaging.[10]

We have previously designed time-resolved variants of displacement assays, which we refer to as supramolecular tandem assays because they are capable of monitoring enzymatic reactions in real time.[11] Interestingly, the assays performed well not only with purified enzymes but also with crude enzyme extracts[11c] and even with dried cells[11a] expressing a particular enzyme. Although the presence of salts, metabolites, and proteins in biological samples may lead to a sizable instantaneous change of the absolute signal owing to a competitive binding,[11c,12] it is the temporal response of the assay that serves as the robust fingerprint of the enzymatic activity, and this temporal response is what is analyzed.[13] This insight has encouraged the development of additional methods in which the spatiotemporal response of indicator displacement can be put to work. For example, we recently introduced tandem membrane assays, in which the reporter pair is spatially isolated inside the liposomes; in this manner, after addition of a target analyte to the outside bulk solution, its protein-mediated translocation through the biomembranes can be monitored in real time.[14] The combined results, namely a sizable tolerance towards competitive binders and a compartmentalized response, encouraged us to proceed towards the penultimate biological challenge: the transfer of IDAs with synthetic receptors to live cells in order to monitor the cellular uptake of biomolecular analytes. In fact, the application of artificial receptors for monitoring bioorganic analytes or drugs in cellular systems has been identified as a challenge in its own right;[1b,4,10,15] it goes beyond the well-established detection of certain inorganic ions in cells[16] or the use of functional group-specific fluorogenic sensors, such as those introduced for biothiols.[17]

We tested various macrocycle/dye combinations for their compatibility with biomembranes and found that the combination of *p*-sulfonatocalix[4]arene (CX4) and *N*,*N*′-dimethyl-9,9′-biacridinium dinitrate (lucigenin, LCG) gave a reporter pair that showed spontaneous uptake into live Chinese hamster ovary (CHO) and fibroblast cells (V79). The uptake of the reporter pair should allow us to subsequently perform cellular IDAs, while the high biocompatibility and low toxicity of both components, LCG and calixarenes in general,[18] as well as CX4 in particular,[18e,f,19] should be beneficial for live-cell measurements. Indeed, incubation with LCG (250 μM, 24 h) had no adverse effect on the cells. The cellular IDA, which we have now been able to realize, is schematically depicted in Scheme 1. The results relating to the successful uptake of analytes, monitored through the switch-on fluorescence response, are shown in Figure 1.

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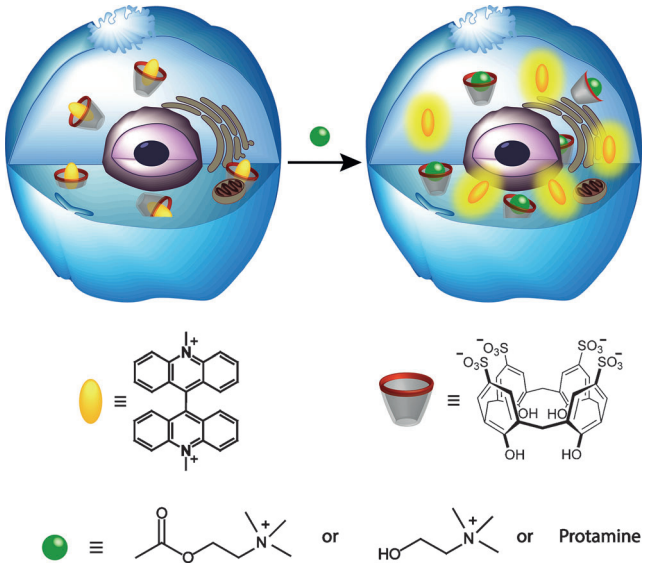

Scheme 1. The uptake of analytes into cells preloaded with the macrocycle–dye complex results in displacement of the dye from the macrocycle and an associated fluorescence increase.

The uptake of dye was confirmed and quantified through incubation of the dye (in the absence and presence of macrocycle), subsequent lysis of the cells, and measurement of the fluorescence after dilution and addition of competitor (to ensure dissociation of the host–dye complex). It should be noted that the fluorescence of the dye is quenched in the supramolecular complex, thus resulting in a switch-off response upon the addition of CX4 and a switch-on response the upon addition of competitor.[20] The uptake of macrocycle (in the presence of dye) was confirmed indirectly from no less than four independent observations: 1) Cells incubated with LCG (800 μM, 30 min) in the presence of CX4 (up to 5-fold excess) displayed an up to twice as efficient uptake of LCG (Table 1),[21] thus suggesting that the host additionally functions as a carrier (reminiscent of DNA transfection with cationic calixarenes).[7] 2) Fluorescence images of cells incubated with LCG in the presence of CX4 afforded much weaker fluorescence (Figure S7 in the Supporting Information). 3) The fluorescence of the lysate of cells incubated with LCG in the presence of CX4 increased substantially upon the addition of competitors, thereby confirming the dissociation of the host–dye complex and thus the presence of CX4 (Figure S4). 4) The observed switch-on fluorescence upon the addition of analytes to the live cells (Figure 1), as opposed to a simple quenching, requires the liberation of the dye from a quenched state, the macrocycle–dye complex.[22]

The surprising finding that the IDA remains operational inside live cells (Figure 1) can be explained by the favorable

Table 1: Uptake efficiency and absolute cellular concentrations of LCG in the absence and presence of CX4.[a]

Cell line	CX4 concentration [mM]	LCG concentration in cells [μM][b]	Uptake [%][b]
V79 cells	0	75	9.4
	4.5	150	19
CHO cells	0	10	1.3
	4.5	20	2.5

[a] Quantified with the lysis method, see the Supporting Information. [b] Incubation conditions: 800 μM LCG, 30 min.

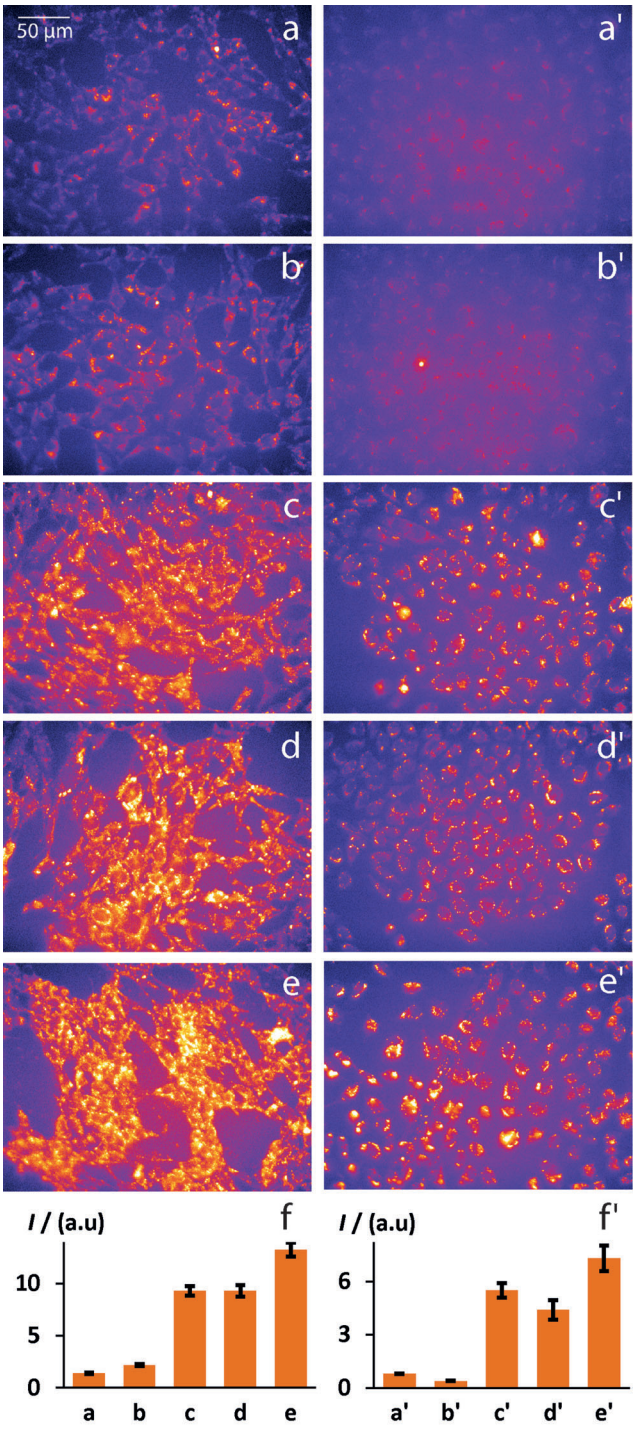

Figure 1. Fluorescence images of V79 cells (left column) and CHO cells (right), both incubated with 50 μM LCG and 250–300 μM CX4 at 37 °C for 15 min, followed by a 10 min incubation with medium (a and a′, as blank controls), 50 mM betaine (b and b′, as negative controls), 50 mM choline (c, c′), 50 mM acetylcholine (d, d′), and 15 min incubation with 200 μM protamine (e, e′). The bar graphs (f, f′) show the relative averaged fluorescence intensity per cell.

characteristics of the specific host/dye system. First, from experiments in pure water, LCG binds CX4 with a high affinity ($K_a = 2.8 \times 10^7\,M^{-1}$)[20] for a synthetic macrocycle,[23] thus facilitating the detection of competitive binders that also display high effective binding constants, such as acetylcholine (ACh, $K_a = 1.0 \times 10^5\,M^{-1}$), choline (Ch, $K_a = 1.0 \times 10^5\,M^{-1}$),[20] and the polycationic peptide protamine ($K_a = 1.24 \times 10^9\,M^{-1}$).[14] Second, CX4 quenches the fluorescence of LCG very efficiently (by a factor of up to 140),[20] which ensures a readily detectable response upon competitive binding. Preliminary experiments in Tyrode's solution and in the media (Figure S6), showed that both properties of the reporter pair are diminished as expected owing to the presence of large amounts of competitive salts, nutrients, and other biomolecules,[12b] but remained at a sufficiently high level ($K_a = 3.0 \times 10^5\,M^{-1}$, fluorescence enhancement factor of 90 in Tyrode's solution and 25 in the media) to retain a response to the target analytes. This encouraged the implementation of the CX4/LCG reporter pair to monitor analyte uptake into live cells.

If the CX4/LCG reporter pair was only physically adsorbed on the cell membrane surface or accessible in the outer cell membrane layer, the incubation of the cells with competitors such as choline, acetylcholine (50 mM),[20] and protamine (200 μM)[14] should lead to an effective release of uncomplexed LCG into the medium because the displacement process itself occurs within milliseconds.[14] However, the analyte solution collected after 10–20 min incubation did not show any LCG fluorescence, thus suggesting that the reporter pair was contained within the cells. By contrast, the cells exposed to the analyte solutions showed significantly enhanced fluorescence, which varied depending on the cell line and the analyte type (Figure 1). These fluorescence enhancements demonstrate that the analytes were taken up within 10–15 min into the cells, where they displaced LCG from the macrocyclic complex to result in enhanced cellular fluorescence. Incubation times longer than 20 min led to relatively smaller fluorescence recoveries, and no significant increase was observed after 5 h of incubation, thus suggesting that the reporter pair proceeds from the cytoplasm into different cellular compartments.[18b] The staining pattern of the released LCG is punctate, in agreement with previous cellular imaging studies of the dye alone.[18d] Although LCG turned out to be photobleached in confocal laser scanning microscopy experiments, the corresponding Z-stacking imaging results (Figure S2) corroborated distribution of the released dye (shortly after analyte addition) through the entire volume of the cell. The fact that dye displacement could also be achieved through cytoplasmic microinjection of the analyte (Figure S8) supports the idea that the initial localization of the CX4/LCG reporter pair is in the cytoplasm.[18a–d]

Indeed, the analytes were selected not only to act as strong competitors, but also because of their efficient cellular uptake. Ch is taken up into non-neuronal cells mainly by specific and nonspecific choline transporters,[24] while ACh crosses the membrane in both directions through organic cation transporters (OCTs),[25] which are expressed in almost all cells.[26] The cellular uptake of protamine is established but mechanistically more diverse, taking place predominantly through rapid endocytosis within 15–30 min,[27] the same time scale as that of the fluorescence recovery in our live-cell IDAs. After endosomal escape,[27b,28] protamine can interact with the reporter pair in the cytoplasm. It should also be noted that betaine was used as a negative control because it is also known to enter cells by a Na^+-dependent active transport mechanism,[29] but does not bind to CX4.[20] Indeed, this analyte (50 mM) afforded no significant fluorescence response in the live cells (Figure 1, parts b and b′), which demonstrates that the method is analyte-selective. The fluorescence enhancements could be semi-quantitatively analyzed (Figure 1, parts f and f′), and the analysis confirmed that, for both cell lines, betaine caused no significant fluorescence response, ACh and Ch caused about the same fluorescence enhancement, and protamine caused the largest fluorescence recovery, even at much lower concentrations. Although the absolute fluorescence responses are composite effects that depend on several factors (including the concentration of the analyte and its binding constant, see above), live-cell-based IDAs in principle offer the possibility for monitoring uptake at different incubation times.

In conclusion, we have established a receptor/dye IDA system that gives an easily measurable response to bioorganic analytes inside live cells. Live-cell-based IDAs could be employed as a simple, economic screening tool to monitor the uptake efficiencies of closely related compounds with similar affinities, such as different trimethylammonium ions or a library of polycationic peptides, the bioactivity of which is presently under intense discussion.

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[21] V79 cells showed more efficient uptake of the dye than CHO cells (Table 1), which could be due, among other factors, to different surface-to-volume ratios of the two cell types (0.65 μm^{-1} for V79 and 0.37 μm^{-1} for CHO cells, see the Supporting Information for size estimations).

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