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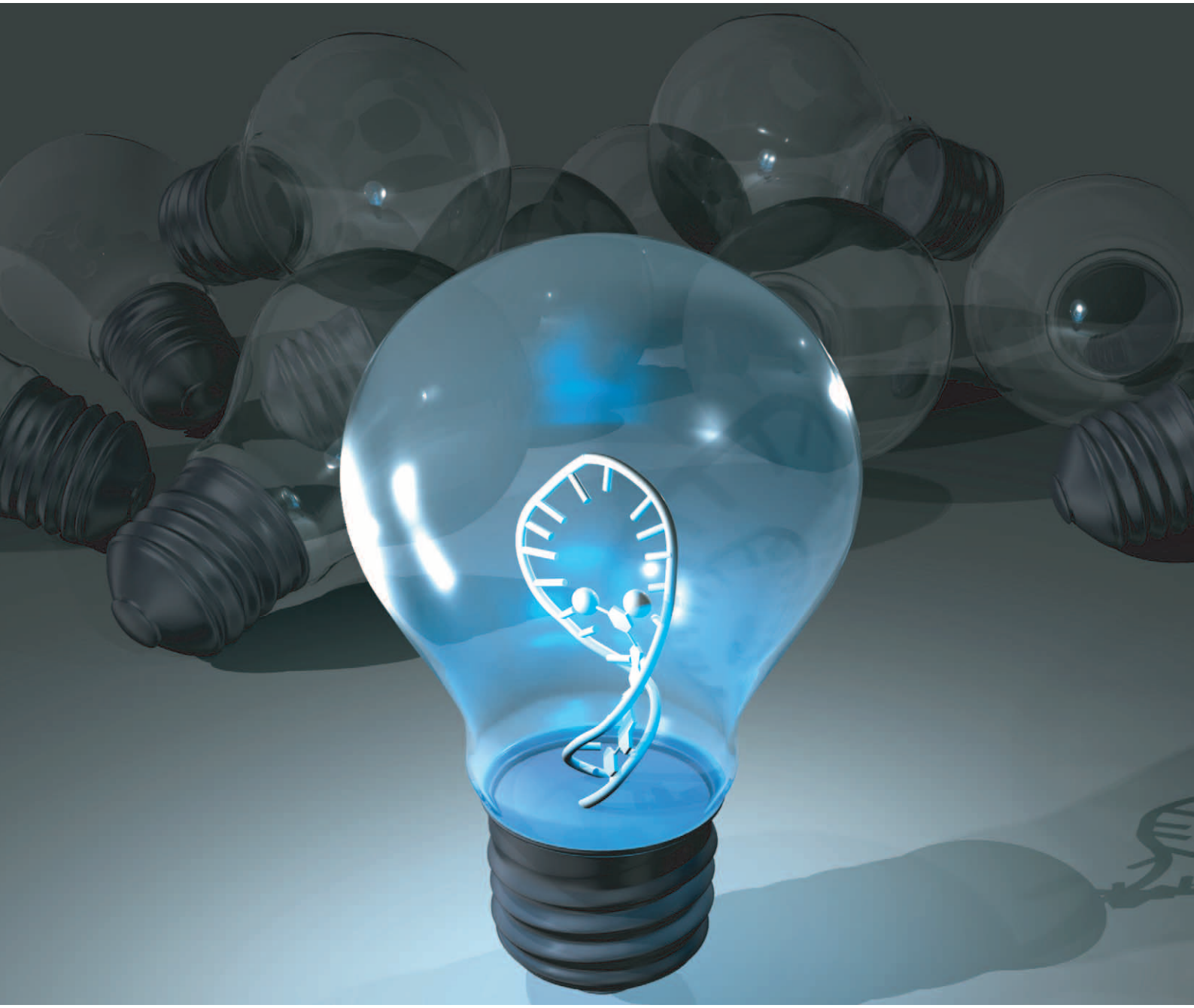

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Transcription monitoring using fused RNA with a dye-binding light-up aptamer as a tag: a blue fluorescent RNA†

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The "light-up" RNA aptamer–Hoechst pair can be used as a fluorescent tag to monitor transcription processes.

Currently, there is much interest in visual monitoring of biological processes. Particularly intriguing targets are RNAs, which play key roles in protein biosynthesis and its regulation. RNA targets may be visualized *via* hybridization to an appropriate probe with a light-up device.[1–3] Another potential yet unexplored approach, the theme of this report, is to use a light-up tag.[4] The potential utility of such an approach is evidenced by the common use of fluorescent proteins such as GFP (green fluorescent protein), which, when fused with a protein, can be used as a fluorescent tag to monitor the protein of interest.[5] The greatest obstacle in applying tag technology to RNA sensing is that fluorescent RNAs do not exist. An alternative method would be to use an RNA tag that binds specifically to and thereby lights up an otherwise nonfluorescent dye.[4] Specific RNA-based binders (aptamers) may be produced using selection/amplification procedures known as SELEX,[6] as was demonstrated by the preparation of a light-up RNA aptamer–fluorophore pair.[7–9] However, despite the high potential of the light-up pairs, no successful demonstration has so far been reported on monitoring of biological processes even under *in vitro* conditions. We recently developed a method to generate systematically a light-up fluorophore–aptamer pair from a microenvironment-sensitive fluorophore using simple SELEX technology. The method is based on the concept that simply selected aptamers can induce change in the microenvironment of a bound dye and thereby enhance fluorescence, and is discussed in a previous report on DNA paired with a modified Hoechst dye.[10] The present report concerns light-up RNA–dye pairs. We describe how RNA aptamers were successfully selected against a modified Hoechst dye, resulting not only in high affinity (K_d = 35 nM) but also in high light-up properties. The aptamer, when fused with luciferase mRNA, can be used as a BFR (blue fluorescent RNA) tag in the presence of the dye to monitor RNA transcription processes.

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† Electronic supplementary information (ESI) available: General, synthesis of Hoechst derivatives, *in vitro* selection, preparation of RNA aptamers, fluorescence and SPR analyses, transcription monitoring, and Fig. S1–S4. See DOI: 10.1039/b808449a

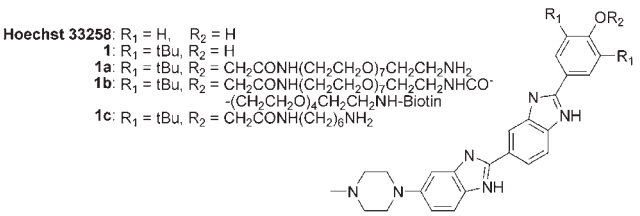

Fig. 1 Chemical structures of Hoechst derivatives.

We carried out *in vitro* selection of RNA aptamers for Hoechst **1**, a derivative of microenvironment-sensitive Hoechst 33258 modified by introducing two *ortho t*Bu groups to suppress its binding affinity to the original AT-rich dsDNA target (Fig. 1).[10–12] Hoechst **1** was converted into Hoechst **1b**, which contains a biotin moiety, using a polyethylene glycol linker and was immobilized on magnetic particles using the streptavidin–biotin interaction. RNA aptamers for immobilized Hoechst **1b** were selected from an RNA pool with a randomized sequence of 31-nt (total length = 71-nt, approximately 10^{15} different initial RNA molecules). After the eighth round of selection, 32 clones were isolated from the enriched pool and sequenced. Twenty-five of the 32 isolated clones could be classified into three types (Classes I, II, and III in Fig. S1 in ESI†).

We first measured enhancement of the fluorescence of the Hoechst derivative.[13] The fluorescence intensity of the otherwise almost nonfluorescent Hoechst **1a** (200 nM, excitation at 345 nm and emission at 470 nm) was enhanced upon addition of the selected aptamers (I_{on}/I_{off} = 3.1–13.1; Fig. S1†).[14] This indicates that the RNA aptamer more or less causes an environmental polarity change for the bound Hoechst **1** derivative, thereby functioning as a light-up trigger in a manner similar to the corresponding DNA aptamers.[10] This is noteworthy because the Hoechst dye was originally specific to AT-rich DNAs and thus is well known as a fluorescent nucleus stainer that has almost no light-up properties against RNAs,[11] indicating the wide applicability of the present aptamer–fluorophore pair-generating strategy. Among the aptamer types, Class II aptamer (200 nM), hereafter denoted aptamer II, exhibited the highest I_{on}/I_{off} ratio (13.1) under these experimental conditions. SPR analysis confirmed a very strong interaction between aptamer II and Hoechst **1b** immobilized on a streptavidin-coated gold surface (dissociation constant, K_d = 35 nM; Fig. S1b†). Therefore, aptamer II was used to develop a blue fluorescent RNA.

To shorten the aptamer sequence, we examined the light-up properties (I_{on}/I_{off}) for Hoechst **1a** of various partial structures

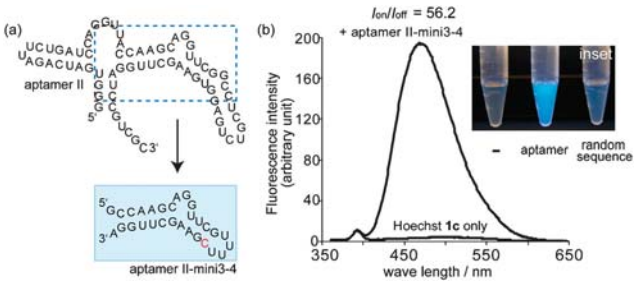

Fig. 2 (a) Predicted secondary structures of aptamer II and II-mini3-4.[15] (b) Fluorescence spectra of Hoechst **1c** (200 nM) in the absence and presence of aptamer II-mini3-4 (200 nM). The fluorescence intensity was measured in the binding buffer (1 × PBS containing 2.5 mM $MgCl_2$) at 25 °C. The inset shows the fluorescence images (excited by a 366-nm transilluminator) of a solution (2 μM) of Hoechst **1c** in the absence (left) and presence of aptamer II-mini3-4 (2 μM, center) or random 29-nt RNA (2 μM, right).

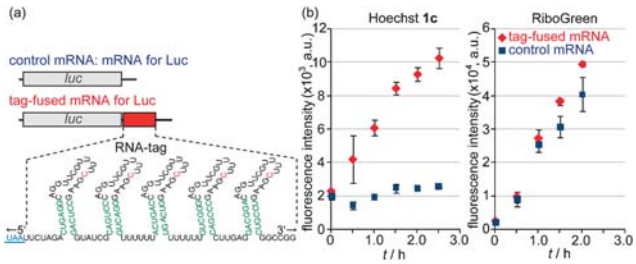

Fig. 3 (a) Control and tag-fused mRNAs for luciferase. The latter contains, after the UAA stop codon (underlined in blue), a tag domain composed of five successive aptamer II-mini3-4 sequences with slight modifications in the tail-stem regions (green) to suppress undesired self-hybridization. (b) Transcription monitoring using a light-up RNA aptamer-modified Hoechst pair (left) and non-sequence specific RiboGreen dye (right). mRNAs were transcribed from dsDNA-templates (6.8 ng μL^{-1}) in transcription buffer containing 7.5 mM NTPs (T7-MEGAscript), and 2.5 μM Hoechst **1c**. After incubation, reaction solution was diluted with buffer and the fluorescence intensity was directly measured with a microplate reader (ex./em. = 355/460 nm and 485/535 nm filter sets for Hoechst and RiboGreen, respectively; experimental details, see supporting information†). Error bars are standard deviations of three experiments.

of the predicted structure (Fig. 2a) of aptamer II (71-nt) (Fig. S2a†), focusing on the stem-loop/bulge domains which would accommodate bulky *t*Bu groups. We produced a substantially shortened (28-nt) sequence (aptamer II-mini3) that had a fluorescence-enhancement ability (I_{on}/I_{off} = 10.4, relative I_{on}/I_{off} = 0.79) similar to that of the original aptamer II (I_{on}/I_{off} = 13.1, relative I_{on}/I_{off} = 1.0). We modified aptamer II-mini3 further *via* addition/exchange of base pairs or deletion/mutation of individual bases (Fig. S2b†), producing aptamer II-mini3-4 (Fig. 2a), which enhanced the fluorescence of Hoechst **1a** with I_{on}/I_{off} = 28.2 (relative I_{on}/I_{off} = 2.15). These results suggest that the sequences of the head-stem and the bulge region of aptamer II-mini3 are sensitively reflected in the light-up properties for the bound dye (Fig. S2b†). Unlike the DNA aptamer,[10] the stem region of the RNA aptamer does not contain successive AU base pairs, as indicated by the low I_{on}/I_{off} value (2.1) for class II-mini3-5, which had an additional AU base pair. I_{on}/I_{off} efficiency was improved further by modifying the dye. The I_{on}/I_{off} values of aptamer II-mini3-4 were sensitive to the side chains of the Hoechst **1** scaffold (Fig. S3† and 2b). Hoechst **1c**, which has a simple aminoalkyl side chain, exhibited the highest enhancement of I_{on}/I_{off} (56.2; Fig. 2b) and the complex had a fluorescence quantum yield of Φ_F = 0.26.[16,17] This high light-up (I_{on}/I_{off}) efficiency enabled easy color visualization of the shortened/optimized aptamer II-mini3-4 against random RNA sequences in the presence of the otherwise almost nonfluorescent imager **1c** (Fig. 2b). Job analysis confirmed 1 : 1 stoichiometry of the **1c**–aptamer complex (Fig. S4†).

Having produced an optimized 29-nt RNA aptamer sequence, we evaluated its utility for mRNA transcription monitoring. We prepared dsDNA-templates for T7 transcription of luciferase mRNA fused or not fused with five successive aptamer II-mini3-4 sequences at the 3′ side of the UAA stop codon (tag-fused mRNA for Luc in Fig. 3a). The tail-side stem regions of the aptamers (shown in green), which were not expected to affect the binding/light-up properties, were partly modified to suppress undesired self-hybridization. The tag-fused mRNA and unfused control mRNA were transcribed from dsDNA templates using T7 RNA polymerase, and fluorescence enhancement of the coexisting Hoechst **1c** was monitored. As shown in Fig. 3b, definite fluorescence enhancement was evident during transcription only for mRNA fused with the RNA tag. In marked contrast, when the transcribed mRNA was detected using a RiboGreen dye, a non-sequence specific fluorescent RNA stainer, the observed fluorescence intensities of both the control and tag-fused mRNAs increased during incubation with almost the same behavior as that of tag-fused mRNA in the presence of Hoechst **1c**, suggesting that Hoechst **1c** was practically specific to the RNA-tag sequence. This indicates that the RNA aptamer selected for the Hoechst **1** derivative can be used as a light-up blue fluorescent RNA tag when fused with a particular RNA.

In conclusion, the optimized aptamer can be fused with luciferase mRNA and the fused RNA as a whole is readily transcribed from the corresponding DNA template with lighting up because of a specific aptamer–dye interaction. Thus, *in vitro* transcription of a particular luciferase gene can be monitored visually. To the best of our knowledge, this is the first successful example of a fluorescent RNA tag (light-up RNA tag–fluorophore pair) that enables transcription monitoring of specific mRNA. This is an important step for production of a fluorescent RNA module, although careful investigation and further experiments are necessary for in-cell application to overcome low but non-zero background fluorescence arising from non-tagged RNAs and dsDNAs.[10] The blue pair can also be used as a light-up probe for label-free aptamer-based fluorescence sensor targeting of proteins or small molecules.[4,18] Combination with other light-up pairs such as fluorescent aptamer–malachite green,[7] DCF–aniline conjugate[8] or fluorogenic cyanine dye[9] pair may even enable multicolor parallel sensing for two or more targets. Further work along these lines is now underway in our laboratory.

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Notes and references

1 For a recent review, see for example: A. P. Silverman and E. T. Kool, *Trends Biotechnol*., 2005, **23**, 225–230 and references therein, including the conventional FISH-based technology.

2 For examples of nucleic acid sensing using a ''light-up/down'' probe, see: (*a*) S. Tyagi and F. R. Kraemer, *Nat. Biotechnol*., 1996, **14**, 303–308; (*b*) M. N. Stojanovic, P. de Prada and D. W. Landry, *ChemBioChem*, 2001, **2**, 411–415; (*c*) R. T. Ranasinghe, T. Brown and L. J. Brown, *Chem. Commun*., 2001, 1480–1481; (*d*) S. Sando and E. T. Kool, *J. Am. Chem. Soc*., 2002, **124**, 2096–2097; (*e*) J. Brunner and R. Krämer, *J. Am. Chem. Soc*., 2004, **126**, 13626–13627; (*f*) J. S. Hartig, I. Grüne, S. H. Najafi-Shoushtari and M. Famulok, *J. Am. Chem. Soc*., 2004, **126**, 722–723; (*g*) J. Cai, X. Li, X. Yue and J. S. Taylor, *J. Am. Chem. Soc*., 2004, **126**, 16324–16325; (*h*) G. T. Hwang, Y. J. Seo and B. H. Kim, *J. Am. Chem. Soc*., 2004, **126**, 6528–6529; (*i*) V. Pavlov, B. Shlyahovsky and I. Willner, *J. Am. Chem. Soc*., 2005, **127**, 6522–6523; (*j*) D. M. Kolpashchikov, *J. Am. Chem. Soc*., 2005, **127**, 12442–12443; (*k*) A. Okamoto, K. Tainaka, Y. Ochi, K. Kanatani and I. Saito, *Mol. BioSyst*., 2006, **2**, 122–127; (*l*) S. Hasegawa, G. Gowrishankar and J. Rao, *ChemBioChem*, 2006, **7**, 925–928; (*m*) D. S. Seferos, D. A. Giljohann, H. D. Hill, A. E. Prigodich and C. A. Mirkin, *J. Am. Chem. Soc*., 2007, **129**, 15477–15479; (*n*) P. Conlon, C. J. Yang, Y. Wu, Y. Chen, K. Martinez, Y. Kim, N. Stevens, A. A. Marti, S. Jockusch, N. J. Turro and W. Tan, *J. Am. Chem. Soc*., 2008, **130**, 336–342; (*o*) D. Baumstark and H.-A. Wagenknecht, *Angew. Chem., Int. Ed*., 2008, **47**, 2612–2614.

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12 For *in vitro* selection of an RNA aptamer against the original Hoechst 33258, see: G. Werstuck and M. R. Green, *Science*, 1998, **282**, 296–298.

13 All of the fluorescence measurements were carried out using 200 nM Hoechst derivative in the absence or presence of 200 nM aptamer in a binding buffer (1 × PBS containing 2.5 mM $MgCl_2$) at 25 °C (excitation at 345 nm). Fluorescence enhancement (I_{on}/I_{off}) refers to the ratio of intensities of a Hoechst derivative (excitation at 345 nm, emission at 470 nm) in the presence (I_{on}) and the absence (I_{off}) of an aptamer after subtracting background fluorescence intensity of the buffer.

14 The initial pool showed only 1.5-fold enhancement in fluorescence of Hoechst **1a**.

15 The RNA secondary structures were predicted using an RNA-structure Version 4.4 program.

16 The quantum yield of Hoechst **1c** (bound) was measured at 25 °C in the presence of a 3-molar excess of aptamer II-mini3-4 in 1 × PBS containing 2.5 mM $MgCl_2$ using 9,10-diphenylanthracene in EtOH ($\Phi_F = 0.95$) as a standard.

17 The quantum yield of free Hoechst **1c** was determined as 0.017 in 1 × PBS containing 2.5 mM $MgCl_2$.

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