Molecular Evolution

International Edition: DOI: 10.1002/anie.201503652
German Edition: DOI: 10.1002/ange.201503652

A Versatile Approach Towards Nucleobase-Modified Aptamers

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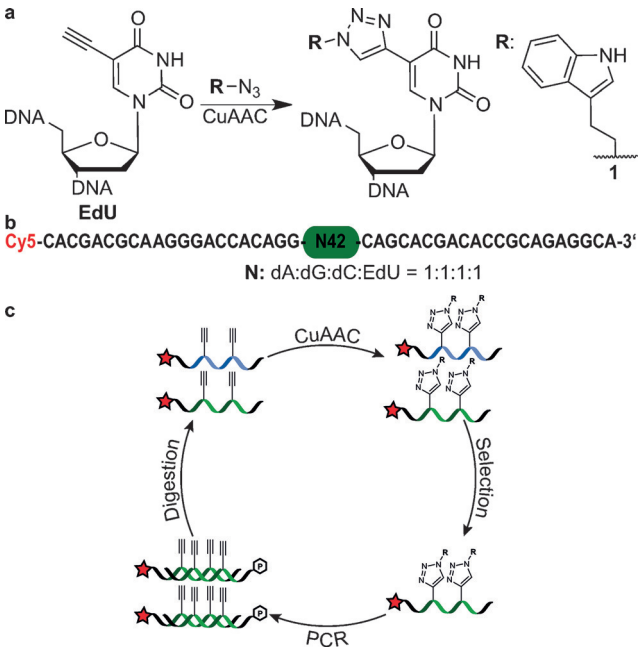
Abstract: *A novel and versatile method has been developed for modular expansion of the chemical space of nucleic acid libraries, thus enabling the generation of nucleobase-modified aptamers with unprecedented recognition properties. Reintroduction of the modification after enzymatic replication gives broad access to many chemical modifications. This wide applicability, which is not limited to a single modification, will rapidly advance the application of in vitro selection approaches beyond what is currently feasible and enable the generation of aptamers to many targets that have so far not been addressable.*

Aptamers are promising tools in life sciences and biomedical research[1] for which there are a myriad of applications.[2,3] Since the first description of SELEX experiments in 1990,[4,5] a plethora of aptamers have been selected against numerous targets, including small molecules[6] and proteins.[7] Some of which have been intensively studied and characterized to allow their implementation in novel diagnostic and therapeutic strategies.[8,9] However, the constrained chemical diversity found in the natural set of canonical nucleotides severely limits the success rates of SELEX experiments.[10] This hurdle can be overcome by expanding the chemical space of the nucleic acid libraries employed for in vitro selection procedures.[11–15] This expansion in functional capabilities is not only beneficial for aptamer selection, but also of great interest for the selection of novel nucleic acid based catalysts.[16,17] Although they are highly desirable and of great interest, methods that allow rapid and modular access to nucleic acid libraries bearing novel chemical entities and are compatible with the enzymatic steps required for in vitro selection experiments are not readily available.[18,19] To date, chemical entities have mostly been introduced during enzymatic replication, thus requiring cumbersome synthesis of the desired building blocks and posing feasibility problems for some desired modifications.[20]

Herein, we describe a novel method, for which we suggest the name click-SELEX, to generate modified nucleic acid libraries by employing copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC or click chemistry).[21,22] First, an alkyne-modified DNA library is prepared by replacing the canonical thymidine building block with C5-ethynyl-2′-deoxyuridine (EdU; Scheme 1 a,b and Figure S1 a–c in the Supporting Information). Second, this library is further modified through click reaction with an azide-containing compound (here with 3-(2-azidoethyl)indole (**1**); Scheme 1 a). A nucleobase-modified DNA library is thereby obtained (Figure S1 c). This library is subsequently subjected to in vitro selection (Scheme 1 c). In this scheme, the modified DNA library is incubated with the desired target molecule, in this case cycle 3 GFP (C3-GFP),[23] immobilized on Co^{2+}-bearing magnetic beads via an N-terminal 6×His-tag moiety (Figure S1 d). C3-GFP was chosen as an initial target owing to its prevalent role in many scientific applications and its fluorescence properties, which allow direct monitoring of its coupling to the selection matrix. To our knowledge, only RNA[24] and no DNA[25] aptamers recognizing GFP have been



Scheme 1. a) CuAAC functionalization of EdU-containing DNA molecules with azides, for example, indole azide **1**. b) Schematic representation of the library design. The 42 nucleotide (nt) random region (N42, green box) containing similarly distributed nucleotides (dA, dG, dC and EdU mixed 1:1:1:1) is flanked by two 21 nt thymidine-free primer binding sites and a fluorescent molecule (Cy5) at the 5′-end. c) Schematic representation of the Click-SELEX process. A synthetic DNA library bearing EdU is modified with an azide-bearing molecule (e.g., **1**) by CuAAC. After incubation with the target molecule and removal of non-bound sequences (selection), the bound sequences are eluted and amplified by PCR with EdU triphosphate instead of thymidine. This step removes the modification in the elongating strand and reintroduces the alkyne moiety. Enzymatic incompatibility problems associated with larger nucleobase modifications are therby avoided. After PCR, the single-stranded DNA is prepared by λ-exonuclease digestion of the 5'-phosphorylated antisense strand (digestion). The modification is then reintroduced by click chemistry (CuAAC) and the obtained library is subjected to the next selection cycle.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201503652.

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described. After incubation, unbound molecules are removed and discarded by washing, whereas the target proteins with the bound modified nucleic acids are eluted through the addition of imidazole. The eluted molecules are then subjected to PCR amplification with C5-ethynyl-2′-deoxyuridine triphosphate instead of thymidine. After amplification, the antisense strand is digested with λ-exonuclease (Figure S1 b), which selectively recognizes and hydrolyses the 5′-phosphorylated strand (introduced by the reverse primer) of the double-stranded DNA.[26] The remaining single-stranded alkyne-modified DNA is again reacted with **1**, thereby reintroducing the indole moiety and reconstituting the starting library for the next selection cycle. To our knowledge, this is the first example were the nucleobase modification is reintroduced in situ after enzymatic replication of the DNA, thus allowing broad access to many chemical modifications and avoiding the enzymatic incompatibility problems associated with larger nucleobase modifications.

After 15 selection cycles (Figure S1 e), we performed interaction analysis by flow cytometry, utilizing the library-inherent 5′-Cy5 fluorophore.[27,28] Briefly, this assay is compatible with the C3-GFP-derivatized magnetic beads used during the selection process and visualizes the amount of Cy5-labeled DNA bound to them. These experiments revealed that a library with enhanced C3-GFP binding properties was obtained (Figure 1 a). The interaction of the enriched library

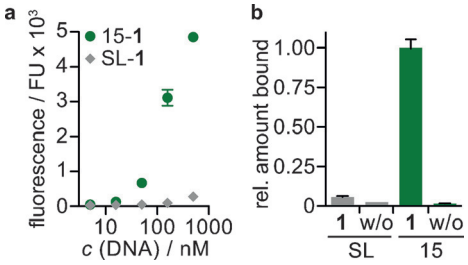

Figure 1. a) Library enrichment. After 15 SELEX cycles, the enriched library (15-**1**) and the start library (SL-**1**) modified with **1** are analyzed for C3-GFP binding by flow cytometry. DNA concentration is plotted against the mean fluorescence intensity. b) The interaction of the enriched DNA library (15, green bars) depends on its functionalization with **1**. The enriched library (15) or the starting library (SL) (500 nM) were incubated with C3-GFP beads, either in unmodified form (w/o, bearing alkyne residues only) or modified with the indole azide (**1**). Mean fluorescence intensity of bound nucleic acid libraries was measured by flow cytometry and normalized to the value obtained for 15-**1**.

was strictly dependent on incorporation of the indole moiety. The non-modified library did not show any binding to C3-GFP and neither did the starting library, independent of its modification status (Figure 1 a,b).

Next, we cloned and sequenced the DNA library from selection cycle 15. We obtained sequences from 36 clones (Figure 2 a and Figure S2 a), with one sequence family dominating the library (Figure 2 a). We investigated the binding behavior of C12-**1**, a representative of the most abundant family, towards C3-GFP by flow cytometry (Figure 2 b). As an essential control, a scrambled variant of C12 (C12sc-**1**), which

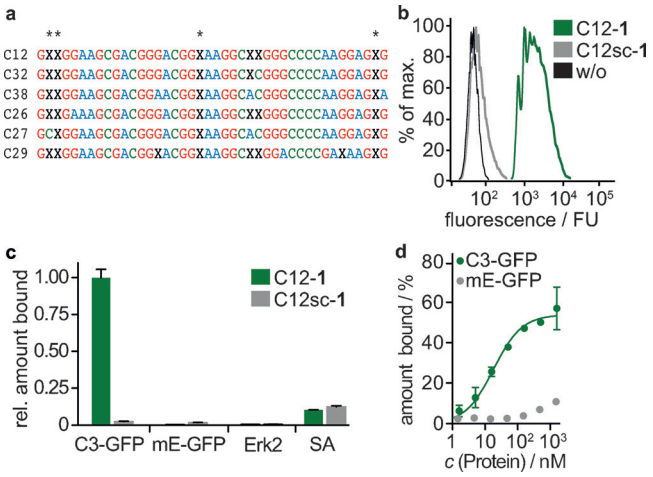

Figure 2. a) Cloning and sequencing results for the DNA library obtained after 15 selection cycles. One dominant family (C12-family) could be identified (see Figure S2 a). Conserved positions for the EdU modification are indicated with an asterisk. b) Analysis of the binding of clone C12 to C3-GFP by flow cytometry. Shown is the amount of Cy5-labelled DNA (500 nM) bound to C3-GFP-bearing beads and beads in the absence of DNA (black curve). C12-**1** (green curve) reveals binding to C3-GFP, whereas the scrambled sequence C12sc-**1** (grey curve) does not. c) Specificity measurements for C12-**1** (green bars). Given is the amount of Cy5-labelled DNA (500 nM) bound to different protein-bearing beads analyzed by flow cytometry. Values were normalized to C12-**1** bound to C3-GFP. C12-**1** does not interact with mE-GFP, Erk2, or Streptavidin (SA). C12sc-**1** (grey bars) does not interact with any protein investigated. d) Filter-retention analysis of C12-**1** with C3-GFP and mE-GFP in solution. For C3-GFP (green curve), a dissociation constant (K_D) of 18.4 ± 3.1 nM was determined.

has the same composition of nucleotides as C12 but in a different order, displayed no interaction with C3-GFP (Figure 2 b). We also tested other members of the C12 family for C3-GFP binding. Briefly, the sequences C32-**1** and C38-**1** showed similar interaction properties with C3-GFP as C12-**1** (Figure S2 b). None of the other clones investigated showed binding to C3-GFP (Figure S2 b).

We next analysed the specificity profile of C12-**1**. To this end, we performed interaction analysis with streptavidin, the mitogen-activated protein kinase ERK2, and the closely C3-GFP-related protein mE-GFP.[29] C3-GFP and mE-GFP differ in only nine point mutations (Figure S2 c,d), of which seven are positioned on the outside of the barrel structure (Figure S2 d). By using flow cytometry, we demonstrated that C12-**1** does not interact with any of these proteins at concentrations 25-fold above its determined K_D value, thus underlining the remarkable specificity of C12 (Figure 2 c, green bars). As expected, the scrambled variant of C12-**1** did not show binding to any of the investigated proteins (Figure 2 c, grey bars). In filter-retention analysis with radioactively labelled molecules, we could show that C12-**1** is able to bind to C3-GFP in solution, independent of the 5′-Cy5 modification, with a dissociation constant (K_D-value) of 18.4 ± 3.1 nM (Figure 2 d).

Subsequently, we further profiled the structure–affinity relationship of C12 with respect to C3-GFP binding based on the nucleobase functionalization. To address this issue, we

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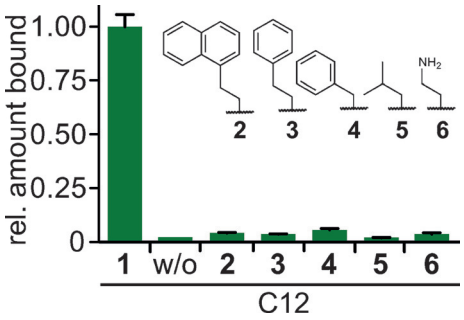

Figure 3. Impact of different functional groups on the binding of C12 to C3-GFP. Given is the amount of Cy5-labelled C12 (500 nM) bound to C3-GFP-bearing beads analyzed by flow cytometry. C12 was used either unmodified (w/o) or after CuAAC-based functionalization with ethylindole (**1**), ethyl-naphthalene (**2**), ethylphenyl (**3**), benzyl (**4**), isobutyl (**5**), or ethylamine (**6**) residues. Values were normalized to indole-functionalized C12-**1** bound to C3-GFP.

synthesized a series of azides (Figure 3, inset) to enable the introduction of 1-ethylnaphthalene (**2**), ethylphenyl (**3**), benzyl (**4**), isobutyl (**5**), and ethylamine (**6**) groups by CuAAC. We then synthesized variants of C12 bearing **2**–**6** (Figure 3 and Figure S3) instead of the indole moiety **1** and analysed their binding behaviour with C3-GFP. Notably, only C12 variants with indole moieties retained binding towards C3-GFP (Figure 3), whereas none of the alternatively functionalized variants maintained binding. This result clearly underlines the dependency of the binding of C12 on the introduction of a defined chemical group. Even very similar aromatic groups, such as **2**, **3**, and **4**, cannot substitute for **1** and recover the binding properties of C12. These data reveal that C12-**1** is a nucleobase-modified DNA aptamer that interacts with C3-GFP. Since the critical indole moiety is introduced by click chemistry, we suggest naming these modified aptamers "clickmers".

Based on in silico predictions, we propose a model for the secondary structure of the C12 family (Figure 4a). However, additional experiments, such as X-ray crystallography and/or NMR spectroscopy, will be needed to elucidate the actual structure. Sequence analysis revealed that EdU at positions 23, 24, 41, and 62 is highly conserved, whereas the positions 47 and 48 are mutated to canonical nucleotides in other C3-GFP-binding members of the C12 family (Figure 2a). We therefore synthesized point-mutated variants of C12-**1** with thymidine residues at position 23 (T23), 24 (T24), 41 (T41), or 62 (T62) and analysed these for binding to C3-GFP. Interestingly, positions 23, 24, and 41 were found to be crucial for the binding properties of C12, since substitution with thymidine led to a complete loss of binding to C3-GFP (Figure 4b). By contrast, placing thymidine at position 62 had no effect on the recognition properties of C12 (Figure 4b), thus indicating that an indole residue at this position is not required to maintain binding.

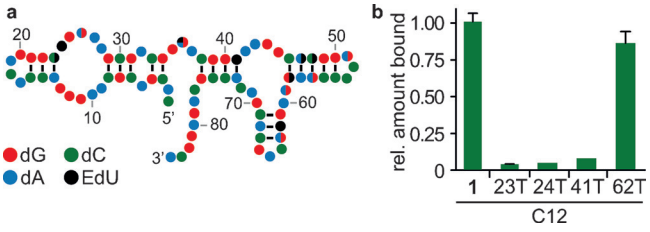

Figure 4. a) In silico predicted model for the secondary structure of the C12 family aptamers. b) Replacing the conserved EdU at positions 23, 24, and 41 with thymidine leads to a loss of affinity. Replacing EdU at position 62 with thymidine had no impact on C12-**1** binding to C3-GFP. Given is the amount of Cy5-labelled and indole-functionalized C12-**1** and single point-mutated variants thereof (500 nM) bound to C3-GFP-bearing beads analyzed by flow cytometry. Values were normalized to fully indole-functionalized C12-**1** bound to C3-GFP.

In summary, we have shown that the use of click chemistry to generate nucleobase-modified DNA libraries gives access to a novel class of modified aptamers, termed "clickmers". Our method retains full compatibility with the common steps of established in vitro selection procedures and was shown to be efficient in yielding an aptamer that recognizes C3-GFP with high specificity. Specific GFP-recognizing DNA aptamers have great potential to be used in several applications in the life sciences, for example, expansion microscopy.[30] The outlined method is not limited to a single functional group but is rather rapidly applicable to a large diversity of chemical entities. This method will give access to a plethora of novel "clickmers" and nucleic acid based catalysts with distinct recognition properties, thus giving researchers access to aptamers targeting molecules and epitopes currently not accessible with traditional in vitro selection processes.[10,31] Furthermore, the described click-SELEX procedure may give access to aptamers modified with chemical entities that are not compatible with solid-phase or enzymatic synthesis conditions for DNA, such as very large or labile entities. It should be mentioned that even with in situ introduction of the modification, the polymerase still has to use the modified strand as a template in the first amplification cycle. Therefore large modifications could still impede enzymatic replication. In this case, we propose a strategy involving a light-cleavable "linker".[32] A light-sensitive moiety, for example, *o*-nitrophenylethyl (NPE), could be placed between the azide and the desired modification, thus allowing removal of the modification prior to enzymatic replication and preserving full compatibility with our method. In future approaches, the method described herein will be expanded to other click reactions, such as norbornene and tetrazine ligation,[33] to enable the orthogonal introduction of multiple modifications. The method could also be combined with other nucleic acid classes, such as native and 2′-modified RNA,[34] locked nucleic acids,[35] or other xeno nucleic acids.[12] The described method does not depend on highly developed organic synthesis or specialized instrumentation and is easily adoptable by the growing field of researchers using aptamers in the life and biomedical sciences, thus satisfying the general demand for specifically tailored aptamers as sophisticated recognition tools[36] and catalysts.[37,38]

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Acknowledgements

This work was made possible by funds from the German Research Council (Deutsche Forschungsgemeinschaft) to G.M. (Ma 3442/4-1). We thank Volkmar Fieberg for providing mE-GFP.

Keywords: aptamers · GFP · molecular evolution · nucleic acids · SELEX

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 10971 – 10974
Angew. Chem. **2015**, *127*, 11121 – 11125

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Received: April 21, 2015
Revised: May 26, 2015
Published online: July 23, 2015

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