ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2020, **56**, 647

Received 4th October 2019,
Accepted 26th November 2019

DOI: 10.1039/c9cc07790a

rsc.li/chemcomm

DNAzyme-powered nucleic acid release from solid supports†

Ting Cao,[abc] Yongcheng Wang,[abd] Ye Tao,[a] Lexiang Zhang,[a] Ying-Lin Zhou,*[c] Xin-Xiang Zhang,*[c] John A. Heyman*[a] and David A. Weitz*[ab]

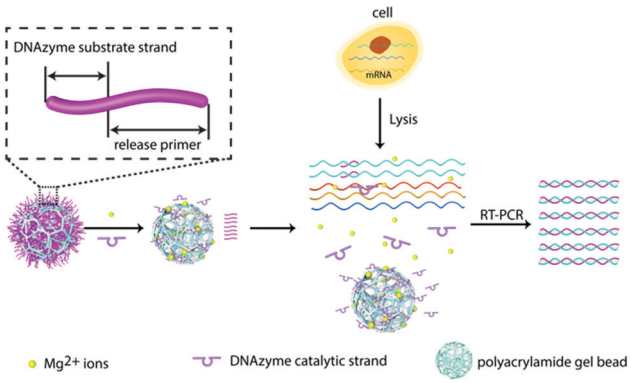
Here, we demonstrate use of a Mg^{2+}-dependent, site-specific DNA enzyme (DNAzyme) to cleave oligos from polyacrylamide gel beads, which is suitable for use in drop-based assays. We show that cleavage efficiency is improved by use of a tandem-repeat cleavage site. We further demonstrate that DNAzyme-released oligos function as primers in reverse transcription of cell-released mRNA.

Numerous molecular-biology techniques require that nucleic acids be captured onto a solid support to enable enrichment or modification, followed by release from the support for subsequent manipulations.[1–3] In a classic example, nucleic acids are captured through specific base-pairing, followed by release using a low-salt elution buffer, as is done to purify polyadenylated RNA through capture onto immobilized polyT-DNA.[4] Alternatively, captured nucleic acids can be released by a restriction endonuclease.[5] In addition, proteases may be used, as in the case of modified DNA.[6] However, the required elution buffers or enzymes may be incompatible with downstream applications and these methods are not suitable if buffer exchange after nucleic acid release is not possible, such as, for example, in methods that use microfluidic droplets for high-throughput compartmentalized assays.[7,8] These issues can be partially overcome through use of a photo-labile linker.[9] In the single cell RNA sequencing (scRNAseq) method inDrop,[10] hydrogel beads decorated with barcoding oligos are encapsulated into nanoliter-sized droplets along with single cells, lysis buffer and reverse transcription reagents. The oligos anneal to mRNA released from the cells and reverse transcription generates barcoded cDNA. During development of this method, it was found that reverse transcription efficiency was dramatically increased by intra-drop release of the oligos from the gels, accomplished through UV-light mediated cleavage of a 1-(2-nitrophenyl)ethyl ester used to link barcoding oligos to the hydrogel beads.[10,11] As a high-throughput single cell mRNA sequencing method based on droplets, inDrop has become a very useful tool to realize scRNAseq without crosstalk between cells by addition of a barcode region with the polyT-primer.[12,13] UV light-triggered primer release is simple and efficient. However, this method suffers from high synthesis costs and the need to protect the beads from light prior to droplet encapsulation. Also, in some applications, UV light treatment may damage molecules of interest.[14,15] Thus, an improved method to cleave nucleic acids from solid support, such as hydrogel beads, would be of great value, particularly for drop-based assays.

In this paper, we present a new method to cleave barcode molecules from solid substrates in a manner that is suitable for use in drop-based assays. We use the inDrop system as our prototypic research target, and we introduce a Mg^{2+}-dependent DNAzyme to release oligos from hydrogel beads to capture cell-released mRNAs.[16,17] DNAzymes, also called DNA enzymes, are DNA oligonucleotides with site-specific DNA-cleavage activity, usually obtained through *in vitro* selection techniques.[18,19] These oligos often require a co-factor, such as metal ions, amino acids or proteins, for cleavage activity,[20–22] and DNAzyme-based biosensors have been developed.[23] This Mg^{2+}-triggered release process can continue as long as DNAzyme strands and Mg^{2+} ions exist in the reaction system. We design and test a tandem dual Mg^{2+}-dependent DNAzyme structure, which shows improved DNA cleavage efficiency. Critically, use of these DNAzymes does not interfere with conversion of captured mRNA into cDNA, or with subsequent PCR amplification. Mg^{2+} ions are compatible with most biological reactions and Mg^{2+}-triggered oligo are released under mild reaction conditions. This DNAzyme-based system is a promising and economical alternative to UV-triggered release

[a] *John A. Paulson School of Engineering and Applied Sciences and Department of Physics, Harvard University, Cambridge, MA 02138, USA. E-mail: jheyman@g.harvard.edu, weitz@seas.harvard.edu*
[b] *Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA*
[c] *Beijing National Laboratory for Molecular Sciences (BNLMS), MOE Key Laboratory of Bioorganic Chemistry and Molecular Engineering, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China. E-mail: zhouyl@pku.edu.cn, zxx@pku.edu.cn*
[d] *Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9cc07790a



Scheme 1 The workflow of DNAzyme-linking primer release and capture for target gene mRNAs.

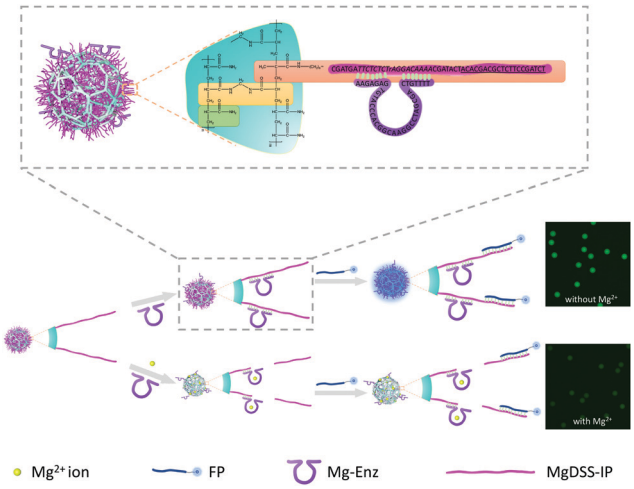


Fig. 1 Rough sketch of polyacrylamide hydrogel beads linked with acrydite-modified DNA oligoes. The molecule structures in different colour background blocks meant different monomers for oligo-linked hydrogel beads; The deoxyribonucleotide bases in italic meant the Mg^{2+}-dependent DNAzyme substrate strand; The deoxyribonucleotide bases in underline meant the random indicator probe.

from solid supports and will also be of general use as a triggerable attachment and cleavage method.

In our DNAzyme-based primer release method, we use a DNAzyme substrate strand to replace the conventional photocleavable organic molecular spacer (Scheme 1). The fabrication process for the polyacrylamide gel beads is the same as that used for the inDrop process, ensuring that we can obtain the same porosity and oligo distribution for the hydrogel beads. With the addition of the DNAzyme catalytic strand and DNAzyme cofactor, the oligos attached to hydrogel beads can be released to the solution because of cleavage of the DNAzyme substrate strand. The gel beads are denser than the fluid and settle to the bottom of the solution, thereby not interfering with the subsequent reaction. The released oligos will capture target-gene mRNAs. After reverse transcription-polymerase chain reaction (RT-PCR), the target mRNAs are transferred into cDNA and further PCR amplification products.

Here, we use a Mg^{2+}-dependent DNAzyme system to perform the oligo release. Magnesium ions are an essential component in the reaction buffer for reverse transcription and are compatible with many biological processes. They do not disturb the subsequent RT-PCR. Also, magnesium ions have a small ionic radius, and can easily diffuse through the pores of the beads.

The Mg^{2+}-dependent DNAzyme substrate strand can be cleaved at the specific adenosine ribonucleotide site in the presence of a DNAzyme catalytic strand and magnesium ions. We use a random DNA sequence to mimic the DNA oligo used in inDrop (a detailed polyacrylamide gel beads generation and characterization is depicted in ESI† section). The DNAzyme substrate strand forms a bridge between this random DNA sequence and the polyacrylamide hydrogel beads through a polymerization reaction with an acrydite-functional group (Fig. 1). The random sequence also acts as an indicator DNA oligo probe (IP) to validate the release process. Another DNA oligo, which is labelled with a FAM fluorophore on the 5′ end, is complementary to the random DNA oligo and is the fluorescence probe (FP). The cleavage characterization flowchart is shown in Fig. 1. For convenience, we label this Mg^{2+}-dependent DNAzyme Substrate Strand-Indicator Probe structure as MgDSS-IP. This Mg^{2+} ion-triggered cleavage process is shown in Fig. S1A (ESI†).

When Mg^{2+} is added to the solution, it permeates into the porous beads, and the Mg^{2+} ions catalyze the cleavage of DNAzyme substrate strand on the hydrogel beads because of the presence of the DNAzyme catalytic strands. Then the random DNA oligo strands, with incomplete DNAzyme substrate strands, will release from the hydrogel bead into the supernatant solution. Discard the supernatant solution and wash the bead three times to remove free oligoes. When the FP is added, hydrogel beads with subsequent DNA sequence exhibit a fluorescence signal while hydrogel beads without MgDSS-IP show no fluorescence signal. We then wash the beads three times and image them with a microscope. The fluorescence intensity of the bead is proportional to the number of DNAzyme strands linking with the bead. And the decrease of the fluorescence intensity of the bead indicates the reduce of the complete DNAzyme strands in the bead. We use the normalized value of fluorescence signal of the hydrogel beads to verify the cleavage efficiency of the DNAzyme structure or the ability to release DNA from hydrogel beads.

For this DNA oligo release process, we explore the influence of several key experimental parameters to gain better knowledge of its cleavage behaviour; these include: reaction temperature, enzyme/substrate strand ratio, magnesium ion concentration and reaction time. We find that the temperature dependence in non-monotonic with an optimum around 20 °C, and with poorer behaviour at temperatures above and below this value, as shown in Fig. 2A. The cleavage efficiency is improved with increasing concentration of enzyme strands relative to the substrate, reaching a plateau at a ratio of 0.5, where about 60% of the oligos are released as shown in Fig. 2B. This catalytic strand only has about forty bases. They can be digested by exonucleases or discarded through purification, which is a common step in many biological processes.[11] The strand cleavage process improves significantly with increasing magnesium ion concentration, above about 0.5 mM, as shown in Fig. 2C. This magnesium concentration

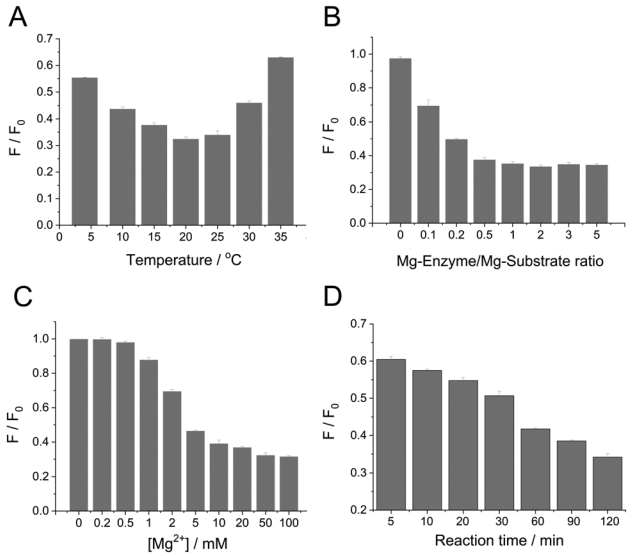


Fig. 2 The influence of different experimental parameters on cleavage process: (A) temperature, (B) Mg-Enz/Mg-substrate ratio, (C) Mg^{2+} concentration, (D) reaction time.

range is compatible with most biological reactions; for example, 1.5 mM Mg^{2+} is used in 1× standard taq reaction buffer for normal PCR and 3 mM Mg^{2+} in 1× first-strand buffer for normal reverse transcription (www.thermofisher.com). Thus, Mg^{2+} ions in the reaction buffer can facilitate the cleavage process without additional magnesium ions. About 40% of the substrate strands are cleaved in the first five minutes during the reaction process and about 60% of the substrate strands are cleaved with 10 mM Mg^{2+} after one hour, and 70% after two hours, as shown in Fig. 2D.

Under the optimized experimental parameters, we obtain about 65% cleavage efficiency for this Mg^{2+}-dependent DNAzyme system in one hour (fluorescent images in Fig. 1). This system is economically more favourable than the conventional UV-triggered DNA oligo release (Fig. S1B, ESI†), and requires organic molecule modification between acrydite-modification and DNA oligo as detailed in Table S1 (ESI†). The traditional method is also less convenient because the experiment must be shielded from UV or bright light to prevent premature cleavage.[11] Moreover, UV light can cause cell damage, such as DNA and RNA mutations,[24–26] which may influence the final results, particularly for DNA or RNA sequencing. By contrast, Mg^{2+}-triggered DNAzyme based cleavage eliminates all these drawbacks. In addition, in the traditional process, UV light triggers inorganic phosphate release from 1-(2-nitrophenyl)ethyl ester with about 58% efficiency.[27,28] By comparison, Mg^{2+} trigger its cleavage process in the first 5 min with about 40% efficiency, and cleavage continues during the whole reaction process, albeit with a decreased speed. An excess of primer loading of the gel beads (about 4E8 primers, calculated according to the experimental section and inDrop protocol) further ensures the successful capture even for the whole transcriptome of the single cell (about 2E5 mRNA in a single mammalian cell[29]).

Even though we believe MgDSS-IP structure satisfies most oligo release process, a still higher cleavage efficiency can be achieved using a tandem design for more general applications. Here, two tandem DNAzyme substrate strands bridge the polyacrylamide hydrogel beads and the DNA release oligos. Provided magnesium ions bind with either of the two DNAzyme strands, the cleavage process will occur spontaneously (Fig. S2, ESI†). We denote the inherent cleavage efficiency of the Mg^{2+}-dependent DNAzyme system as k; then, the DNA oligo release efficiency for the single MgDSSIP structure is nearly equal to k. For the TDMgDSS-IP structure, the DNA oligo release efficiency is $1 - (1 - k)^2 = k(2 - k) > k$. Thus, this tandem design can indeed improve the oligo release efficiency compared with the initial MgDSS-IP structure (a comparation cleavage result is shown in Fig. S3, ESI†).

We also test the influence of the experimental parameters for this tandem DNAzyme system at 20 °C. The cleavage efficiency reaches a plateau value of 80% when the ratio of the concentration of enzyme strand to substrate strand is 1.0 (Fig. S4, ESI†). Compared with the ratio value 0.5 in the single DNAzyme-linking hydrogel bead system, the tandem DNAzyme system requires more enzyme strands because of the additional substrate strand structure. Also, the magnesium ion concentration has a larger influence on the cleavage process; 20 mM Mg^{2+} can catalyze almost 80% of the cleavage of the tandem DNAzyme substrate oligoes, as shown in Fig. S5 (ESI†). The system exhibits the same cleavage tendency as that of the single DNAzyme system, except that the tandem DNAzyme system has a higher cleavage efficiency for the same reaction time as shown in Fig. S6 (ESI†). The cleavage efficiency is about 82% after two hours.

Here, we verify the ability of these TDMgDSS-IP hydrogel beads to capture mRNAs in cells. We use the GAPDH gene as a model and find that it satisfies the DNA oligo release requirement in mRNA capture. TDMgDSS–GAPDH hydrogel beads are incubated with Jurkat cells in sample A as shown in Fig. 3. As a control, sample A_0 has the same reaction conditions as sample A but does not include any cells. TDMgDSS–GAPDH primers without hydrogel beads are used in samples B and B_0. A random sequence linking GAPDH primers (Adaptor-GAPDH) without hydrogel beads, which acts as a positive control, is used in sample C and C_0. Their detailed sequences are listed in Table S1 (ESI†). From Q-PCR data, we find that A, B and C samples all give a positive result. T_m curves are further demonstrating formation of the PCR amplification product (Fig. S7, ESI†). Also, in A, B and C samples, new DNA bands with 100–200 base pairs appear in Agarose gel electrophoresis (Fig. S8, ESI†). Notably, no additional magnesium ions are added; the magnesium ions in the commercial working buffer are sufficient for the cleavage process. Further, we prepare a new buffer removing magnesium ions from the solution to test if magnesium ions dominate substrate strand cleavage and trigger the next RT process. As in Fig. S9 (ESI†), the solution without magnesium ions cannot produce amplification produce (Fig. 2C and Fig. S5, ESI† also show that the cleavage reaction of the DNAzyme system will not occur without magnesium ions). Thus, these tandem DNAzyme linking hydrogel beads are very effective in capturing mRNAs in cells and hold great promise in the DNA oligo release process.

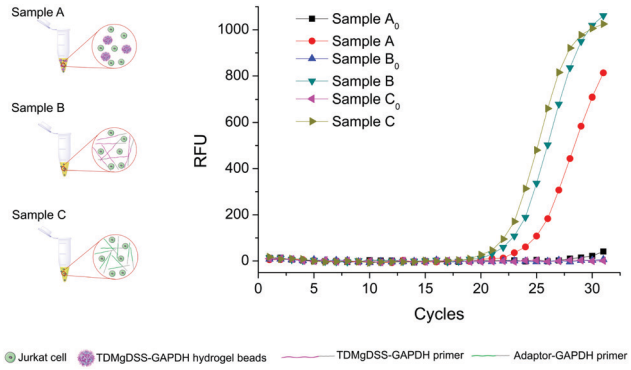


Fig. 3 Q-PCR data of different reverse transcription primer treatments for GAPDH gene cDNA amplification: samples A_0, A, B_0, B, C_0 and C. Except for having no cells, sample A_0, B_0, and C_0 had the same reaction condition with sample A, B and C, respectively. In samples A and A_0, TDMgDSS-IP hydrogel beads were used to primer reverse transcription. In sample B and B_0, TDMgDSS–GAPDH primers without hydrogel beads were used. In sample C and C_0, Adaptor-GAPDH primers without hydrogel beads were used.

Mg^{2+} ion is a cofactor for all DNA polymerases, including reverse transcriptase.[30] This Mg^{2+}-dependent DNAzyme powered nucleic release also has a great potential in genome and transcriptome analysis, particularly for drop-based single cell sequencing assay. It reserves high biocompatible ability, high loading efficiency for per drop and high primer loading ability and, at the same time, resolves the problem of high experimental cost, troublesome light-avoided experimental operation and uncertain influence on the nucleic acid sequence information caused by the conventional photo-cleavable organic molecule in inDrop for the hydrogel beads.[10,11]

In conclusion, oligos released from supports are used in numerous molecular biology processes. Using the inDrop technique as a model, we here construct a Mg^{2+}-triggered DNAzyme-based oligo release from hydrogel beads. This DNAzyme linker modification is much more economical and can be easily synthesized and stored. In addition, the hydrogel beads with the added DNA sequences are more stable and convenient to use, especially compared with the conventional photo-cleavable linker. We also report a tandem DNAzyme linker structure that improves the DNA oligo release efficiency and shows favorable cleavage behaviour for mRNA capture in cells with shorter reaction time and improved cleavage efficiency. It is an improved way to promote DNA oligo release from the hydrogel beads through the cleavage of DNAzyme strands. This work provides a new means for DNA oligo release from solid supports. It is a generally applicable method for triggered release of DNA from solid supports.

This work was supported by the Harvard MRSEC under NSF award DMR 1420570, by the NSF (DMR-1708729) and by the NIH (R01EB023287). This work was also supported by Wyss Institute. T. C. was supported by the Fellowship from the China Scholarship Council (CSC, File No. 201706010062).

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 R. R. Breaker and G. F. Joyce, *Chem. Biol.*, 1994, **1**, 223–229.
2 J. Kim, J. P. Hilton, K. A. Yang, R. Pei, M. Stojanovic and Q. Lin, *Sens. Actuators, A*, 2013, **195**, 183–190.
3 H. P. J. Buermans and J. T. den Dunnen, *Biochim. Biophys. Acta, Mol. Basis Dis.*, 2014, **1842**, 1932–1941.
4 F. Ozsolak and P. M. Milos, *Nat. Rev. Genet.*, 2011, **12**, 87–98.
5 W. H. Chen, G. F. Luo, Y. S. Sohn, R. Nechushtai and I. Willner, *Adv. Funct. Mater.*, 2019, **29**, 1–12.
6 J. K. Awino, S. Gudipati, A. K. Hartmann, J. J. Santiana, D. F. Cairns-Gibson, N. Gomez and J. L. Rouge, *J. Am. Chem. Soc.*, 2017, **139**, 6278–6281.
7 D. J. Shin, Y. Zhang and T. H. Wang, *Microfluid. Nanofluid.*, 2014, **17**, 425–430.
8 C. Ziegenhain, B. Vieth, S. Parekh, B. Reinius, A. Guillaumet-Adkins, M. Smets, H. Leonhardt, H. Heyn, I. Hellmann and W. Enard, *Mol. Cell*, 2017, **65**, 631–643.
9 G. Leriche, L. Chisholm and A. Wagner, *Bioorg. Med. Chem.*, 2012, **20**, 571–582.
10 A. M. Klein, L. Mazutis, I. Akartuna, N. Tallapragada, A. Veres, V. Li, L. Peshkin, D. A. Weitz and M. W. Kirschner, *Cell*, 2015, **161**, 1187–1201.
11 R. Zilionis, J. Nainys, A. Veres, V. Savova, D. Zemmour, A. M. Klein and L. Mazutis, *Nat. Protoc.*, 2017, **12**, 44–73.
12 E. Hedlund and Q. Deng, *Mol. Aspects Med.*, 2018, **59**, 36–46.
13 X. Zhang, T. Li, F. Liu, Y. Chen, J. Yao, Z. Li, Y. Huang and J. Wang, *Mol. Cell*, 2019, **73**, 130–142.
14 E. J. Wurtmann and S. L. Wolin, *Crit. Rev. Biochem. Mol. Biol.*, 2009, **44**, 34–49.
15 R. P. Rastogi, Richa, A. Kumar, M. B. Tyagi and R. P. Sinha, *J. Nucleic Acids*, 2010, **2010**, 592980.
16 R. R. Breaker and G. F. Joyce, *Chem. Biol.*, 1995, **2**, 655–660.
17 S. Liu, C. Cheng, H. Gong and L. Wang, *Chem. Commun.*, 2015, **51**, 7364–7367.
18 R. R. Breaker, *Nat. Biotechnol.*, 1997, **15**, 427–431.
19 M. Hollenstein, C. Hipolito, C. Lam, D. Dietrich and D. M. Perrin, *Angew. Chem., Int. Ed.*, 2008, **47**, 4346–4350.
20 S.-F. Torabi, P. Wu, C. E. McGhee, L. Chen, K. Hwang, N. Zheng, J. Cheng and Y. Lu, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 5903–5908.
21 R. M. Kong, X. B. Zhang, Z. Chen, H. M. Meng, Z. L. Song, W. Tan, G. L. Shen and R. Q. Yu, *Anal. Chem.*, 2011, **83**, 7603–7607.
22 M. M. Ali, S. D. Aguirre, H. Lazim and Y. Li, *Angew. Chem., Int. Ed.*, 2011, **50**, 3751–3754.
23 C. E. McGhee, K. Y. Loh and Y. Lu, *Curr. Opin. Biotechnol.*, 2017, **45**, 191–201.
24 Y. Lu, N. Yamagishi, J. Miyakoshi, A. Noda, T. Yagi and H. Takebe, *Carcinogenesis*, 1996, **17**, 2343–2345.
25 C. A. Billecke, M. E. Ljungman, B. C. McKay, A. Rehemtulla, N. Taneja and S. P. Ethier, *Oncogene*, 2002, **21**, 4481–4489.
26 K. Takeshita, J. Shibato, T. Sameshima, S. Fukunaga, S. Isobe, K. Arihara and M. Itoh, *Int. J. Food Microbiol.*, 2003, **85**, 151–158.
27 J. H. Kaplan, B. Forbush and J. F. Hoffman, *Biochemistry*, 1978, **17**, 1929–1935.
28 R. S. Given, P. G. Conrad, A. L. Yousef and J. Lee, *Synthetic Organic Photochemistry*, CRC Press LLC, 2003, pp. 1–46.
29 E. Shapiro, T. Biezuner and S. Linnarsson, *Nat. Rev. Genet.*, 2013, **14**, 618–630.
30 T. A. Steitz, *J. Biol. Chem.*, 1999, **274**, 17395–17399.

