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REVIEW

Current approaches for RNA-labelling to identify RNA-binding proteins[1]

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Abstract: RNA is involved in all domains of life, playing critical roles in a host of gene expression processes, host-defense mechanisms, cell proliferation, and diseases. A critical component in many of these events is the ability for RNA to interact with proteins. Over the past few decades, our understanding of such RNA–protein interactions and their importance has driven the search and development of new techniques for the identification of RNA-binding proteins. In determining which proteins bind to the RNA of interest, it is often useful to use the approach where the RNA molecule is the "bait" and allow it to capture proteins from a lysate or other relevant solution. Here, we review a collection of methods for modifying RNA to capture RNA-binding proteins. These include small-molecule modification, the addition of aptamers, DNA-anchoring, and nucleotide substitution. With each, we provide examples of their application, as well as highlight their advantages and potential challenges.

Key words: RNA-binding proteins, biotin labelling, digoxigenin labelling, aptamer labelling, affinity capture, nucleotide-substitution method.

Résumé : L'ARN est impliqué dans tous les domaines de la vie, jouant un rôle clé dans nombre de processus d'expression génique, de mécanismes de défense de l'hôte, dans la prolifération cellulaire et les maladies. Une composante importante de plusieurs de ces manifestations consiste en la capacité de l'ARN à interagir avec les protéines. Au cours des quelques dernières décennies, notre compréhension de telles interactions ARN–protéines et de leur importance a stimulé la recherche et le développement de nouvelles techniques visant l'identification de protéines liant l'ARN. Afin de déterminer quelles protéines se lient à un ARN d'intérêt, il est souvent utile d'utiliser une approche où la molécule d'ARN sert d'appât, lui permettant de capturer les protéines d'un lysat ou d'une autre solution pertinente. Les auteurs font ici la synthèse d'une série de méthodes pour modifier l'ARN afin de capturer des protéines liant l'ARN. Celles-ci comprennent la modification de petites molécules, l'ajout d'aptamères, l'ancrage d'ADN et la substitution de nucléotides. Pour chacune, ils fournissent des exemples de leur application et soulignent leurs avantages et défis potentiels. [Traduit par la Rédaction]

Mots-clés : protéines liant l'ARN, étiquetage à la biotine, étiquetage à la digoxigénine, étiquetage d'aptamères, capture d'affinité, méthode de substitution de nucléotides.

Introduction

Over the past several decades, great strides have been made in characterizing the roles of many types of RNA-binding proteins. This is due to the development of newer technologies, and the growing body of researchers that are working towards understanding these interactions. **R**NA-**b**inding **p**roteins (RBPs) form **ribo**nucleo**p**rotein (RNP) complexes with single-stranded or double-stranded RNA by typically recognizing a specific sequence and (or) structure (Arnez and Cavarelli 1997; Lunde et al. 2007; Cléry et al. 2008; Linder and Jankowsky 2011), as well as through unconventional binding modes as recently reviewed by Hentze et al. (2018). Different domains can be present on a variety of RBPs that allow for the recognition of RNAs, some of these include: **R**NA **R**ecognition **M**otifs (RRM), **K**-**h**omology domains (KH), RGG (Arg-Gly-Gly) boxes, zinc fingers, **d**ouble-**s**tranded **R**NA-**b**inding **d**omains (dsRBD), or DEAD-box (Asp-Glu-Ala-Asp) helicase domains (Chen and Varani 2005; Lunde et al. 2007; Cléry et al. 2008; Glisovic et al. 2008; Valverde et al. 2008; Linder and Jankowsky 2011).

Although these RBPs have been known to play important cellular roles by interacting with mRNAs (Gerstberger et al. 2014; Dixit and Lukeš 2018; Moore and von Lindern 2018), recent work has demonstrated the importance of RBPs and their interactions with various types of noncoding RNA such as microRNAs, small nucleolar RNAs, long-noncoding RNA, piRNA, and viral RNA (Bagni and Lapeyre 1998; Jiang and Coller 2012; Ciafrè and Galardi 2013;

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Falaleeva et al. 2016; Zealy et al. 2017; Grzechnik et al. 2018; Lei et al. 2018; Tedeschi et al. 2018; Baou et al. 2011; Zhou et al. 2018; Qiao et al. 2019; Yang et al. 2019). The formation of RNP complexes acts to regulate essential cellular functions from cell division to cell death, transcription to post-translational gene regulation, and downstream functions (Clayton and Shapira 2007; Glisovic et al. 2008; Kishore et al. 2010; Brinegar and Cooper 2016). It is important to note that the majority of these RNAs are regulated by cellular proteins, and the dysregulation of some of these RNAs has been implicated in diseases of the cardiovascular, neurological, and immune systems, as well as cancer (Lukong et al. 2008; Esteller 2011; Brinegar and Cooper 2016; Conlon and Manley 2017; Newman et al. 2017; Li et al. 2018; Zhou et al. 2019). Thus, the identification of RBPs and their modes of interaction could allow researchers to elucidate how fundamental cellular processes are governed.

To investigate the interactions between an RNA of interest and RBPs within a cellular environment, researchers often start off with simpler in-vitro models, owing to the complexity of the cellular environment (Das et al. 2012). Within this approach, T7 RNA polymerase is typically employed to in-vitro transcribe linearized DNA to produce the RNA of interest. The RNA produced is then purified using size-exclusion chromatography under the nondenaturing conditions to maintain a native fold of the RNA of interest (Kieft and Batey 2004; Easton et al. 2010; Kanwal et al. 2018). Such monodispersed in-vitro transcribed RNA can then be modified via a number of approaches for use in identification of their binding partners. Here, we review the application of small-molecules (e.g. biotin labelling), aptamers, nucleotide substitution, and DNA anchoring that can be used to label the RNAs of interest to detect their protein binding partners. With each method, we also discuss some of the advantages and disadvantages pertaining to their use. The key highlights of each method have also been summarized in Table 1.

Small-molecule modifications

Small-molecule modification is a popular RNA-labelling method, where small molecules can be immobilized on a particular substrate, followed by an application of crude sample (e.g., cell lysate) containing a mixture of proteins to perform pull-down assays for identification of specific RBPs. Typical small-molecule tags used are biotin, desthiobiotin, or digoxigenin (Fig. 1, Table 1). Using this approach has the overall advantage of simplicity, low cost, and appears to have minimal influence on the RNA secondary structure necessary for binding (McCreery 1997; Kresoja-Rakic and Felley-Bosco 2018).

Biotin labelling

Biotin labelling of RNA is one of the most widely used nonradioactive methods for determining RNA-binding proteins (Figs. 1A and 2) (Moritz and Wahle 2014; Liu et al. 2015; Lindehell et al. 2015; Rinaldi et al. 2015). The two main types of RNA biotin-labelling methods are internal labelling and 5′ or 3′ end-labelling. Internal labelling relies on the use of a specific biotin labelled **n**ucleotide **trip**hosphate (NTP) during an in-vitro transcription (Panda et al. 2016). Internal labelling of the target RNA with biotin has the potential downside of RNA misfolding due to steric hindrance, or may result in synthetic RNA–protein complexes that are not observed intracellularly (Jazurek et al. 2016). Thus, here we focus on the 3′ or 5′ end labelling methods that are added after **in-v**itro **t**ranscription (IVT). In end-labelling approach, the 3′ or 5′ terminus of the RNA is tagged with a single molecule of biotin (Figs. 1A and 2) and then incubated with cell lysate to allow the RNA to form complexes with cellular proteins (England et al. 1980; Keith 1983). The biotinylated RNA and associated RNA-binding proteins are then pulled down using streptavidin beads, for which biotin has a high-affinity ($K_d \sim 6.5 \times 10^{-14}$ mol/L) (Piran and Riordan 1990; Holmberg et al. 2005; Panda et al. 2016). A similar pull-down method has been used, employing biotinylated apo-B mRNA to purify several mRNA-associated proteins, including the complementation factor involved in APOBEC-1-dependent RNA editing (Mehta and Driscoll 1998).

The biotin–streptavidin interaction is one of the strongest known noncovalent biological interactions, and provides an efficient capture of potential RBPs. Moreover, the biotin–streptavidin complex has been demonstrated to sustain a wide range of buffer conditions, including different salt concentrations, heat, pH, and proteolytic conditions, thus offering versatility to the method (Chaiet and Wolf 1964; Hofmann et al. 1980; González et al. 1997, 1999; Stayton et al. 1999; Laitinen et al. 2006). Typically, mass spectrometry is used downstream to identify proteins that could interact with the biotin-labelled RNA (Jazurek et al. 2016). Once identified using mass spectrometry, the selected proteins are expressed and purified to validate their interactions with RNA using methods such as **e**lectrophoretic **m**obility **s**hift **a**ssays (EMSA) and filter binding assays.

There are several benefits to using biotin to label RNA. First, this method is very sensitive, requires less time, is easy-to-use, and is cost-effective when compared with the other methods (Moritz and Wahle 2014). Moreover, the 3′ or 5′ biotin end-labelling method can be used for RNA probes from 22–1600 nucleotides (nt) long while causing a minimal disturbance in RNA secondary structure (Moritz and Wahle 2014; Paingankar and Arankalle 2015). It is also important to note that although this method can be used to label longer RNAs, the biotin labelling incubation times may increase, and labelling efficiency decreases as RNA probes increase >450 nt, or have a complex structure (O'Connor et al. 2005; Piskounova et al. 2008). Another shortfall is the very high-affinity interaction between biotin and streptavidin, which requires proteins associated with biotin-labelled RNA to be eluted under harsh denaturing conditions, which affects the re-usability of the streptavidin beads (Holmberg et al. 2005). However, the interaction between streptavidin and biotin can also be broken under nondenaturing conditions by incubating the column in a non-ionic aqueous solution at temperatures above 70 °C for a short period of time (Holmberg et al. 2005). An additional consideration when using this method is that because biotin is a cofactor for many carboxylases in the cell, such as pyruvate carboxylases, there may be false positives with proteins that bind biotin directly (Tytgat et al. 2015).

Desthiobiotin labelling

Desthiobiotin is a non-sulfur-containing analog of biotin (Fig. 1B) and an alternative to biotin labelling (Hirsch et al. 2002). Desthiobiotin has a lower affinity for biotin capture proteins, such as avidin and streptavidin (K_d 6.6×10^{-10} mol/L) (Levy and Ellington 2008). Although in principle the pull-down method to identify RNA-binding protein is very similar to that of biotin labelling, the lower affinity to streptavidin exhibited by desthiobiotin allows for less harsh elution conditions to release the RNA with RNA-bound proteins (Jazurek et al. 2016). For example, the complexes can be eluted using desthiobiotin or a biotin solution to displace the desthiobiotin-bound RNA (Hirsch et al. 2002). Desthiobiotin labelling has been used in a variety of applications, from malignant cell lines, to lentiviral vector applications to identify RNA-binding proteins (Chen et al. 2010; Kresoja-Rakic and Felley-Bosco 2018).

Digoxigenin labelling

Digoxigenin (DIG) is another popular small-molecule being used for the labelling of RNA to identify RNA-binding proteins (Fig. 1C). DIG was developed primarily as a way to detect nucleic acids and proteins without the use of radioactivity (McCreery 1997). There are several ways RNA can be DIG-labelled, such as internal labelling through PCR incorporation of rUTPs, nick translation, or direct chemical labelling (McCreery 1997). However, these methods could possibly interrupt RNA structure, and cause

Table 1. Advantages and disadvantages of the various RNA labelling methods for detecting RNA-binding proteins.

Label	Labelling position	RNA modification length (nt)	Binding partner	K_d (mol/L)	Pros	Cons	References
Small molecule modifications							
Biotin	5′ or 3′ ends	n/a	Streptavidin beads	$\sim 10^{-14}$	High affinity, easy to use, and cost effective	Chemical treatments to clean the streptavidin beads	Piran and Riordan 1990
Desthiobiotin	5′ or 3′ ends	n/a	Streptavidin beads	6.6×10^{-10}	Less harsh conditions needed compared with biotin	Lower affinity than biotin	Levy and Ellington 2008
Digoxigenin	Internal, and 5′ or 3′ ends	n/a	Streptavidin beads	$\sim 1.6 \times 10^{-8}$	Fewer false positives compared with biotin	Lower affinity than biotin	McCreery 1997
Aptamer approaches							
PP7	5′ or 3′ ends	25	PP7 coat protein	$\sim 10^{-9}$	Works with varying pH and ionic strengths conditions	Production of a coat protein	Lim and Peabody 2002
S1	5′ or 3′ ends	47 × 4 repeats	Streptavidin	$\sim 70 \times 10^{-9}$	Streptavidin used for capturing, and not coat protein	Resin is expensive, and fewer binding sites compared with Sephadex™	Leppek and Stoecklin 2014
D8	5′ or 3′ ends	33	Sephadex G-100 and G-200	n/a	Sephadex G-200 is cheap; urea can be used to elute	Potential for false positives, and less affinity than S1	Srisawat et al. 2001
Tobramycin	5′ or 3′ ends	40	Tobramycin-modified beads	5×10^{-10}	Antibiotic used to elute	Sepharose beads are expensive	Jiang and Patel 1998
Streptomycin	5′ or 3′ ends	46	Streptomycin-coupled Sepharose	$\sim 10^{-6}$	Antibiotic used to elute	Lower K_d (~1 μmol/L) compared with tobramycin (5 nmol/L)	Wallace and Schroeder 1998
MS2	5′ or 3′ ends	19 × 6–8 tandem repeats	MS2 coat protein	4×10^{-9}	Coat protein used to elute	Requires making a coat protein	Lykke-Andersen et al. 2000; Yoon et al. 2012
Csy4 (H29 A)	5′ or 3′ ends	20	Csy4 nuclease	50×10^{-12}	High affinity Fewer false positives	Production of a functional Csy4 mutant	Faoro and Ataide 2014 Lee et al. 2013
Mango	5′ or 3′ ends	23–31	Thiazole orange (TO) or TO1-biotin	3.2×10^{-9}	Can characterize low abundance proteins from cells	Quadruplex can be tedious to make and label	Xia et al. 2017; Trachman et al. 2017
Other							
Nucleotide Substitution (BrUTP)	Internal	n/a	Anti-BrdU antibody	n/a	Washes do not require denaturing conditions	Changes in charge distribution Requires large amount of starting material	Beach and Keene 2008
Ribotrap (DNA anchoring)	5′ or 3′ ends	n/a	Complementary DNA “arm”	Base pairing	Entire experiment can be kept at 4 °C	Weakly bound proteins difficult to identify	Liu and Sun 2005

Fig. 1. Small molecules typically used to label RNAs to identify RNA-binding proteins. Structure of (A) biotin, (B) desthiobiotin, (C) digoxigenin.

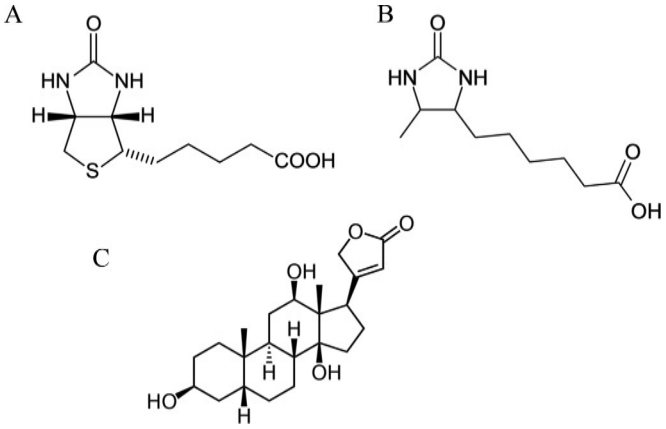
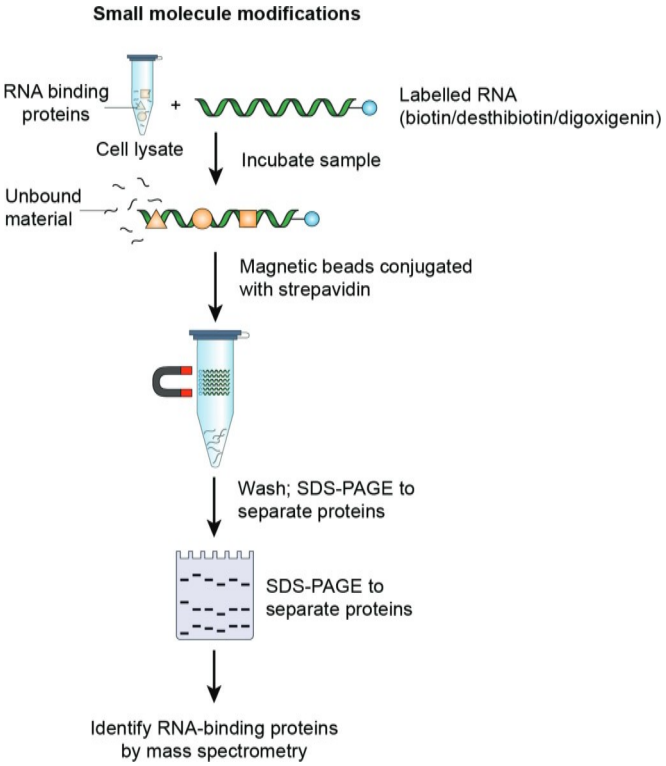


Fig. 2. An outline of the method for capturing ribonucleoprotein complexes using small molecules. The labelled RNA is incubated in cell lysate to allow RNA-binding proteins (RBPs) to interact with the RNA. Biotin and desthiobiotin require streptavidin-coated magnetic beads, whereas the DIG-labelling protocol employs anti-DIG antibodies conjugated onto magnetic beads. They are added to the cell lysate and RNA mixture, then the unbound material is washed away while a magnet holds the material of interest. The components are then separated by SDS–PAGE, and the RBPs are identified by mass spectrometry. [Colour online.]



either nonspecific binding to RBPs or prevent binding. A solution to this issue is to perform DIG end-labelling at the 3′ terminus of the target RNA. For this reaction to occur, the enzyme terminal transferase catalyzes the addition of a single DIG labelled dideoxy-UTP to the 3′ mRNA end (Fig. 2) (Tomassi et al. 2017). The DIG-labelled RNA probe can then be incubated with cellular extract, and then placed to incubate onto magnetic beads. These magnetic beads are conjugated with a chemiluminescent anti-DIG antibody that is used to immunoprecipitate the potential RBPs associated with the DIG-labelled RNA (Booy et al. 2018). Herranz and Pallás (2004) demonstrated, using DIG-labelled riboprobes, that the movement protein interacts with the *Prunis necoritic ringsport* viral ssRNA.

The main advantage of DIG over biotin labelling is the minimization of false-positive RBPs. Where biotin is found rather ubiquitously in many cell types, digoxigenin is a steroid produced specifically in *Digitalis* plants and does not appear to have confounding interactions with RNA–protein identification in other species (McQuaid et al. 1995). Moreover, DIG hybridization is fast, relatively cheap and sensitive, and with less background noise during detection (Viterbo et al. 2018).

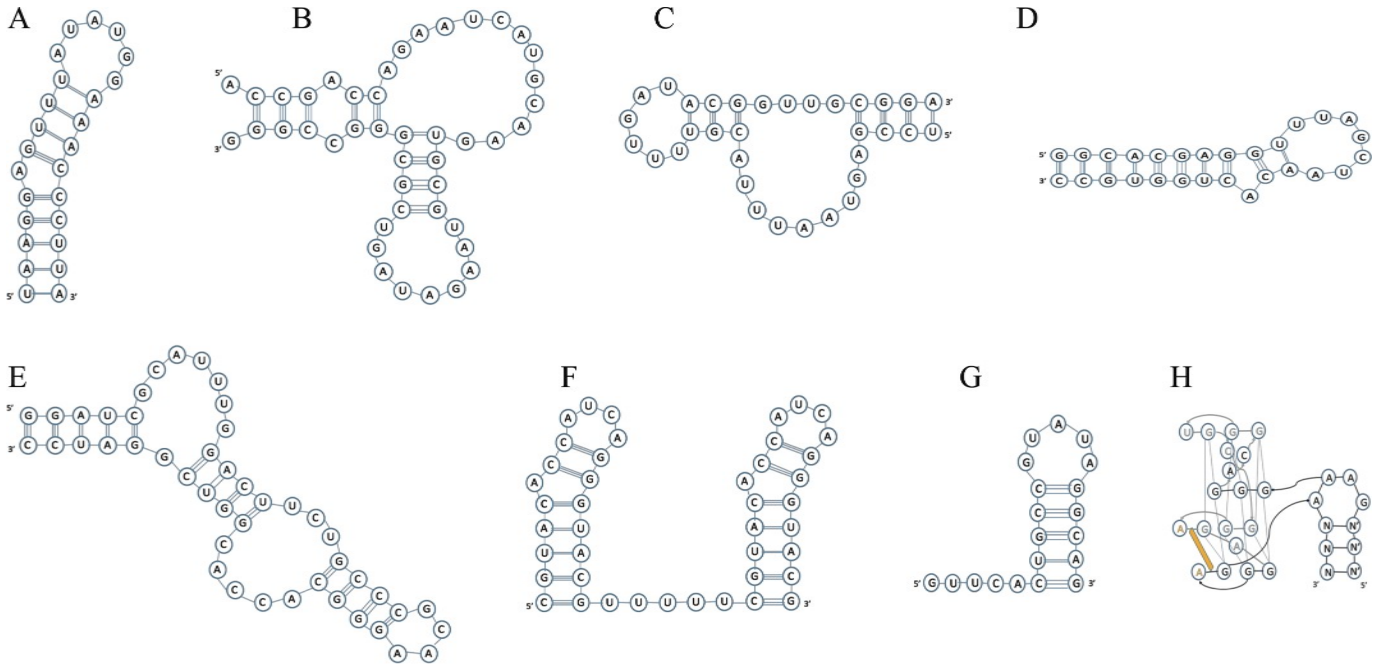
Aptamer modifications

In addition to small molecules, the application of RNA aptamers is another popular strategy for modifying RNA to identify RBPs (see Table 1). An RNA aptamer is a folded oligonucleotide that interacts with a target molecule (Fig. 3). RNA aptamers interact with their respective substrates via electrostatic and hydrophobic interactions along their folded structure (i.e., stem loops, etc.) enabling anchoring (Ni et al. 2011; Stoltenburg et al. 2016; Sun et al. 2016; Mallikaratchy 2017). The process of determining aptamers and the substrates they bind to, is known as the **s**ystematic **e**volution of **l**igands by **ex**ponential enrichment (SELEX) (Mallikaratchy 2017). SELEX involves generating a library of oligonucleotides and screening each for binding interactions with a bait molecule (i.e., small molecule, protein, oligonucleotide) (Ellington and Szostak 1990; Tuerk and Gold 1990). Once interactions are identified, the oligonucleotides are cloned, sequenced, and can then be used to anchor the bait RNA for future capture assays (Keefe et al. 2010). In its first application, the technique was used to identify a high-affinity RNA that interacts with the bacteriophage T4 DNA polymerase (Tuerk and Gold 1990). Since then, aptamers have been identified that have affinities for their ligand that are comparable to antibody–antigen affinities (Johansson et al. 1997; Lim et al. 2001; Demirci et al. 2013; Bjerregaard et al. 2016). One advantage of using the aptamer approach is that they can be designed and directly incorporated into the RNA of interest during in-vitro and in-vivo transcription, without the requirement of additional kits or chemical modifications (Keefe et al. 2010). The most commonly used RNA aptamers are PP7, S1, D8, tobramycin, streptomycin, MS2, Csy4 (H29A), and Mango (Figs. 3 and 4) (Bachler et al. 1999; Ward et al. 2011; Marchese et al. 2016). More recently developed aptamers such as Csy4, which is based on the CRISPR-nuclease Csy4 system, and the fluorescence-based quadruplex aptamer, Mango, are also discussed. When considering labelling with an aptamer, it is important to understand that aptamers can change the native structure of the RNA, which in turn could prevent the binding of RBPs (Faoro and Ataide 2014). While there are no strict rules on where the aptamer can be placed within the RNA molecule, incorporation at the 5′ or 3′ ends are the most logical sites to minimize the potential distortions in shape and charge distributions.

PP7 aptamer

The PP7 aptamer (Fig. 3A) was identified using the SELEX, and was shown to interact with the PP7 protein coat of the *Pseudomonas aeruginosa* bacteriophage, with high affinity ($K_d \sim 1$ nmol/L) (Lim et al. 2001; Lim and Peabody 2002). It was demonstrated that the phage protein coat interacts with its own RNA genome to assist in controlling translation (Lim et al. 2001). Researchers have used this approach to study the RNA-binding proteins involved in the small nucleolar RNA 7SK, which has been shown to play a role in regulating eukaryotic transcription (Hogg and Collins 2007). This experiment was performed by expressing the PP7 coat protein with an N-terminal fusion of two Protein A domains separated by

Fig. 3. Schematics of the aptamers that are being used to label RNA molecules: (A) PP7; (B) S1; (C) D8; (D) tobramycin; (E) streptomycin; (F) MS2; (G) Csy4 (H29A); and, (H) Mango. [Colour online.]



linker for cleavage by the **t**obacco **e**tch **v**irus TEV) protease. The PP7 coat protein contains two binding epitopes: one for the RNA aptamer, and one for the associated antibody. The RNA with the PP7 aptamer is incubated with PP7 coat protein to allow for binding, followed by addition of the cellular lysate and an additional incubation step (Fig. 4). Next, a resin containing anti-PP7 antibodies is used to capture the PP7-bound RNA along with its RBPs. The unbound cell extract is washed away, and the complexes are eluted using TEV protease to cleave the linker (Hogg and Collins 2007). A second purification step can be performed using the tobramycin aptamer by incubating it in agarose derivatized with tobramycin (Hogg and Collins 2007). Elution was then performed using tobramycin in excess. The strength of this approach is that this technique can work with a wide range of ionic strengths and pH (Carey and Uhlenbeck 1983).

S1 aptamer

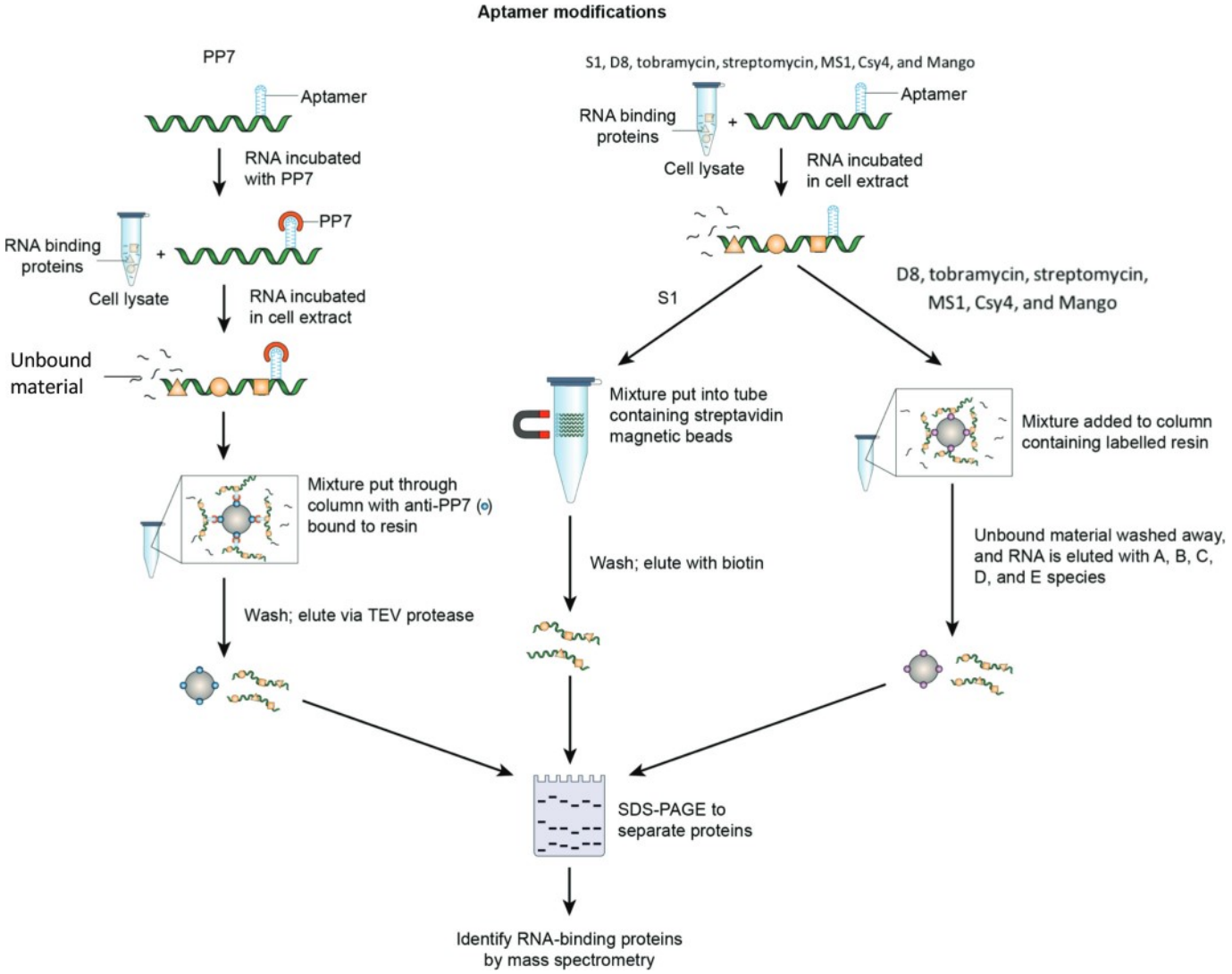
The S1 aptamer (Fig. 3B) has also been identified through SELEX and shown to interact with streptavidin. Dong et al. (2015) performed a pull-down assay with the dengue virus 5′ and 3′ **un**translated **r**egion (UTR) by modifying the UTRs' 3′ end with an S1 aptamer, and used streptavidin linked to magnetic beads (Dong et al. 2015). The RNA and the proteins bound to it were then eluted using biotin that competed with the streptavidin binding site (Fig. 4). The RBPs were then identified using mass spectrometry. S1 was also shown to bind with high-affinity to streptavidin when optimized four tandem repeats are introduced on the RNA for optimal RBP capture (Leppek and Stoecklin 2014). Comparing one S1 aptamer vs. four repeats, Leppek and Stoecklin (2014) observed a 15-fold increase in the captured RNA. However, when using six repeats Iioka et al. noticed a loss in efficiency (Iioka et al. 2011). The four-tandem repeat was shown to bind to streptavidin with a K_d of ~70 nmol/L, but with a slight modification of S1, the K_d of the new S1 (S1m) changed to 29 nmol/L (Leppek and Stoecklin 2014). It is important to note that while using the S1 aptamer, cellular biotin must be blocked to have available streptavidin binding sites to ensure efficient capture of the RNA, therefore, avidin is incubated in cellular extracts prior to putting the RNA with the S1 aptamers into the cell extract. The avidin interacts with cellular biotin, which frees up more of the streptavidin binding sites when performing the pull-down assay (Srisawat and Engelke 2002). One advantage of using the S1 aptamer over MS2 and PP7 is the readily available streptavidin that can be used for capturing, i.e., recombinant coat proteins are not required to be made or synthesized for experimentation.

D8 aptamer

Similar to the aptamers described above, the D8 RNA sequence (Fig. 3C) was also identified using the SELEX, and was demonstrated to bind to Sephadex® G-100 and G-200, which are commonly used matrices in gel filtration that employ cross-linked dextran B512 with epichlorohydrin (Srisawat et al. 2001; Srisawat and Engelke 2002; Walker et al. 2008; Ravelet and Peyrin 2009; Slobodin and Gerst 2010). One of the benefits of the D8 aptamer is that its interaction with such matrices is highly specific (Srisawat et al. 2001; Walker et al. 2008). The potential RBPs attached to the RNA of interest can be eluted with either using a denaturing condition (8 mol/L urea) or using a nondenaturing condition through applying the competitive binder dextran B512 (Srisawat et al. 2001).

Sephadex G-100 and G-200 are cost-effective alternatives compared with S1 streptavidin magnetic beads (Srisawat et al. 2001). They are also very stable and can be regenerated and re-used several times (Walker et al. 2008). Another benefit to the use of this aptamer is that large amounts of cell extract can be used to purify RNA. Additionally, and importantly, the rapid, mild elution conditions make this method very useful for weaker protein–RNA interactions, as well as for preserving native protein and RNA structures during elution (Srisawat et al. 2001). One disadvantage of using the D8 aptamer is its lower affinity for its matrix compared with that of the streptavidin-binding RNA aptamers mentioned above (Srisawat and Engelke 2002). This leads to a balance whereby employing multiple washes results in a loss of D8-bound RNA, but insufficient washes produce more background contamination. The D8 aptamer has been used to identify RNaseP ribozyme from yeast (Srisawat and Engelke 2002; Walker et al. 2008) as well as study the protein interactions of the noncoding regions of polioviruses (Flather et al. 2016).

Fig. 4. Aptamer-based methods for the identification of RNA-binding proteins (RBPs). The left side of the image shows the experimental design for the PP7 aptamer using the PP7 coat protein to bind to the aptamer, followed by incubation with cell lysate. Finally, the sample is incubated in resin conjugated to anti-pp7 antibodies allowing the antibody to bind to the PP7 coat protein. Washing of the unbound material is followed by elution with tobacco etch virus (TEV) protease cleaving the linker. The middle of the image illustrates the S1 aptamer procedure, which uses streptavidin magnetic beads for capturing the ribonucleoprotein (RNP) complexes. Unbound material is washed away while the magnetic beads hold the streptavidin–RNP complex. Samples are eluted using biotin in excess. Finally, the right side of the image shows the D8, tobramycin, streptomycin, MS2, Csy4 (H29A), and Mango aptamers. The resin is labelled with the corresponding capture entity, and the eluant differs according to the aptamer [8 mol/L urea or dextran B512 (D8); tobramycin (tobramycin aptamer); streptomycin (streptomycin aptamer); MS2 coat protein (MS2); imidazole (Csy4); and biotin (Mango)]. After the elution step, each of these methods proceeds to an SDS–PAGE to separate the RNA-binding proteins, followed by protein identification by mass spectrometry. [Colour online.]



Tobramycin aptamer

Tobramycin is an antibiotic that interacts with the 30S and 50S **r**ibosomal **RNA** (rRNA) on the ribosome, preventing the 70S complex from forming prior to translation, which consequently causes cell death in bacteria (Yang et al. 2006). The tobramycin-binding aptamer was identified using SELEX (Fig. 3D), which has a dissociation constant of 5 nmol/L with tobramycin (Jiang and Patel 1998). The RNA-labelling assay involves attaching the tobramycin aptamer to the 3′ end of adenovirus-derived MINX pre-mRNA. Using this aptamer, researchers identified a number of proteins involved in RNA splicing, including U1 and U2 snRNP proteins along with non-snRNP splicing factors (Hartmuth et al. 2002).

Streptomycin aptamer

Similar to tobramycin, streptomycin (Fig. 3E) is also an antibiotic. Streptomycin binds to the 30S ribosomal subunit of bacteria, interfering with **t**ransfer **RNA** (tRNA) binding, and causing in codon misreads and ultimately cell death (Sharma et al. 2007; Demirci et al. 2013). The streptomycin aptamer was found using affinity chromatography with streptomycin-coupled Sepharose™ beads and a pool of nucleotide fragments (Fig. 4) (Wallace and Schroeder 1998). The K_d of the streptomycin aptamer with streptomycin is ~1 μmol/L, which is lower than the tobramycin dissociation constant. Using this method, spliceosomal U1A protein was purified from yeast cell extract, small nuclear RNA, yeast group II intron-binding proteins, viral RNA binding proteins, and bacterial ncRNA-binding proteins, and the bacteriophage coat protein MS2 (Windbichler and Schroeder 2006).

MS2 aptamer

The MS2 aptamer is a 19-nucleotide RNA hairpin structure derived from the MS2 RNA bacteriophage (Fig. 3F) (Johansson et al. 1997;

Carrier et al. 2016). The bacteriophage MS2 coat protein interacts with high specificity and affinity to the MS2 RNA hairpin aptamer (Johansson et al. 1997). Capitalizing on this strong interaction, multiple tandem repeats (typically runs of 6–8 aptamers) are incorporated into the 3′ end of the RNA of interest, enabling efficient pulldown using immobilized MS2 coat protein (Lykke-Andersen et al. 2000; Yoon et al. 2012). With either in-vitro or in-vivo RNA transcription, the MS2 labelled RNA is incubated with cellular extracts or cellular lysate and applied to solid amylose resin (Fig. 4). The resin contains an immobilized fusion protein consisting of the maltose binding protein and the MS2 coat protein (Zhou and Reed 2003; Slobodin and Gerst 2010). Next, the MS2-labelled RNA and associated RBPs are eluted using excess MS2 coat protein, and the samples can be analyzed using mass spectrometry (Lim and Peabody 2002; Said et al. 2009). The MS2 aptamer has been used to isolate highly stable RNPs such as U1 snRNP as well as less stable mRNPs and RNPs associated with noncoding regulatory RNAs (Lykke-Andersen et al. 2000).

CRISPR endoribonuclease Csy4

A more recently discovered method of RNA modification is that of tagging with an engineered CRISPR Csy4 endoribonuclease aptamer. Csy4 endoribonuclease is a component of the prokaryotic adaptive immune system, **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats (CRISPR), where the nuclease cleaves guide RNA transcripts bearing a specific hairpin sequence (Fig. 3G) (Lee et al. 2013). The rationalization for this approach was based on the discovery that a single histidine mutation (H29A) in the enzyme prevents RNA cleavage activity, but that the activity can be readily reversed upon addition of imidazole (Lee et al. 2013). Thus, once the Csy4-aptamer-containing RNA is immobilized on the mutant Csy4 endonuclease resin and all unbound cellular components washed away, imidazole can be added, which takes the place of the missing histidine in the active site. This reactivates the enzyme and Csy4 proceeds to cleave the RNA strand, enabling elution of the RNA along with its accompanying RNA-binding proteins. Similar to the above aptamer methods, the subsequent determination of the bound proteins can be undertaken using mass spectrometry (Lee et al. 2013).

A benefit of this approach is that with the high-affinity binding of the aptamer to the Csy4 nuclease ($K_d \sim 50$ pmol/L), more stringent washes can be performed, thereby reducing the background contamination and chances of false positives (Faoro and Ataide 2014; Lee et al. 2013). The inherent gain in purity via this method removes the need for gel purification before mass spectrometry analysis. Authors claim the mutant Csy4 is a readily produced and robust enzyme; however, it would still need to be produced, labelled, and incorporated into beads before it could be used (Lee et al. 2013). Other downsides to this approach are that weaker-bound RNA-binding proteins may be washed away by the more stringent washes and therefore lost for identification. This approach has been used to identify microRBPs in different cell lines (Lee et al. 2013), and adaptations of the approach are used in other molecular biology methods (Liang et al. 2017).

Mango aptamer

The RNA Mango aptamer (Fig. 3H) was designed to provide another option for affinity-based native purification methods of associated RNPs with the added benefit of fluorescence detection (Panchapakesan et al. 2017). This RNA aptamer consists of a 19 nt fluorophore-binding domain that has a high affinity (K_d 3.2 nmol/L) to fluorophore reagents: **t**hiazole **o**range (TO), TO1-biotin, or TO1-desthiobiotin (Trachman et al. 2017; Xia et al. 2017). The RNA of interest is synthesized by in-vitro transcription with the Mango aptamer tag, and is then incubated in the cellular extract to allow RNP complexes to form (Panchapakesan et al. 2017). The extract containing the Mango-tagged RNA is incubated in streptavidin solid support resin conjugated to TO1-desthiobiotin. Desthiobiotin is used because of its lower binding affinity to streptavidin (K_d 6.6 × 10^{-10} mol/L) when compared with biotin (K_d 10^{-14} mol/L) (Piran and Riordan 1990; Levy and Ellington 2008). The resin is then washed to remove any nonspecific interactions, and the Mango-aptamer–RNA–RNPs are eluted using excess free biotin. The RNP complex can be visualized using SDS–PAGE analysis, further purified using size-exclusion chromatography, and RNA-binding protein identity can be confirmed using liquid chromatography – mass spectrophotometry (LC-MS). Downstream characterization studies between the RNA and RBP can also utilize the fluorescent tag by using single-molecule fluorescence cross-correlation spectroscopy, or other methods (Panchapakesan et al. 2017).

The benefits of this method are that the reactions can be performed under native conditions and with similar equipment that is used during DIG labelling (i.e., streptavidin beads, and desthiobiotin). Moreover, the high affinity interaction between Mango and thiazole orange promotes efficient pull-down of RNP complexes in nanomolar concentrations with low volumes of cell extract. This approach requires a fluorophore, whereas the other aptamer methods do not require that additional label. This does give the researcher an added tool to track the elution of the aptamer complexed with RNA-binding proteins, but does require more preparation steps.

Nucleotide substitution

Another method for RNA anchoring is the application of nucleotide substitution. In this approach, one of the four nucleotides, typically **u**racil **t**riphos**p**hate (UTP) is modified with a heavy metal and incorporated into the RNA during in-vitro transcription (Fig. 5, Table 1) (Beach and Keene 2008; Faoro and Ataide 2014). Monoclonal antibodies to this modified uracil can be used to pull-down the RNA. The most common nucleotide substitution method for this purpose is the RiboTrap (Beach and Keene 2008).

RiboTrap

RiboTrap is carried out in-vitro by transcribing the RNA of interest in the presence of 5-bromo-UTP (BrUTP, BrU) free nucleotides (Beach and Keene 2008). The BrU-labelled RNA is then incubated with cellular lysate. Following incubation, the BrU–RNA–RBP complexes are captured by anti**br**omo**d**eoxy**u**ridine (BrdU) monoclonal antibodies that are conjugated to the solid resin (Faoro and Ataide 2014). Different washes with varying stringencies can be performed to analyze weak or tightly bound RBPs. Elution of the BrU–RBPs is with excess BrdU. The pulled-down samples can be run on an SDS–PAGE, and the RBPs can be identified using mass spectrophotometry (Beach and Keene 2008).

The advantages of this method are that the washes are performed under nondenaturing conditions, and the 5-bromo-UTP RNA modification can minimize nonspecific binding (Faoro and Ataide 2014). Moreover, this labelling technique can also be used in-vivo by transfection of the BrU–RNA into yeast hosts, bacteria, and mammalian cell culture to identify RBPs in nuclear and (or) cytosolic extracts (Beach and Keene 2008; Han et al. 2014). The caveat to this modification is that the charge distribution is changed with the substitution of a bromine for the hydrogen in the uracil structure. This, in turn, changes the shape of the molecule and could hinder any native conformations that the RNA possesses. Furthermore, this method requires large amounts of starting material to ensure the detection of RNA–protein interactions (Michlewski and Cáceres 2010).

DNA anchoring

RNAs of interest can also be anchored with the complementary strands of DNA immobilized to a medium for identification of RBPs (Torres et al. 2018; Liu and Sun 2005). This approach employs the natural binding affinities of RNA and DNA strands and can incorporate annealing and dissociation temperatures for the cap-

Fig. 5. The nucleotide substitution approach to identify RNA-binding proteins (RBPs). The uracil triphosphates are replaced with 5-bromo-UTP during an in-vitro transcription process of the RNA of interest. The modified RNA is then incubated with cell lysate to allow RBPs to interact with the RNA. The potential RNA–RBP complexes are captured with an anti-BrdU antibody that is conjugated to resin. The mixture is loaded onto the column containing an appropriate resin, followed by a washing step to remove unbound components, and an elution with excess BrdU to obtain RBPs that can be identified using mass spectrometry. [Colour online.]

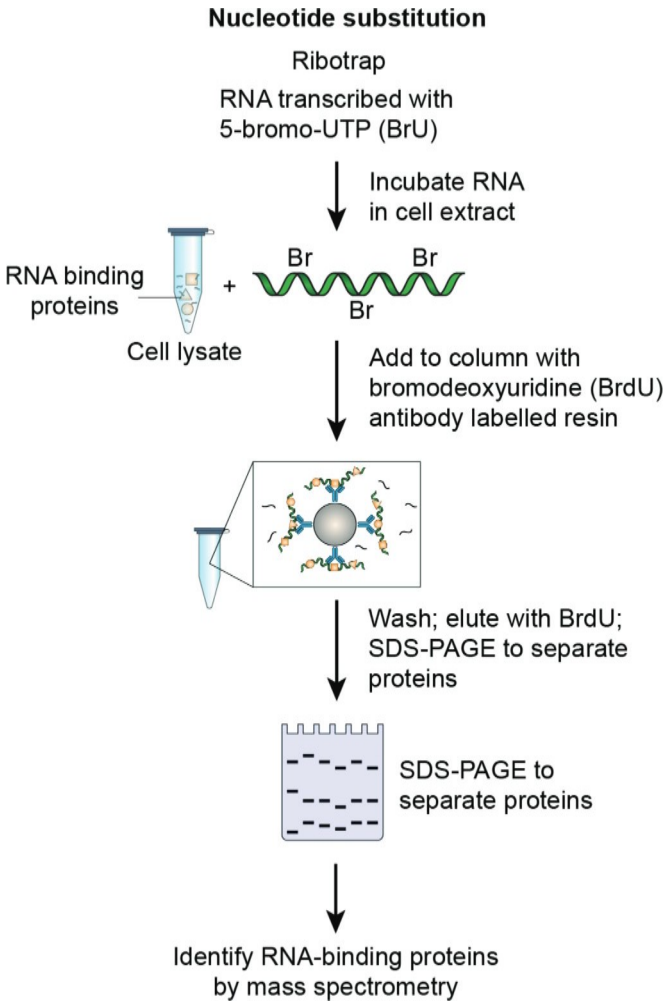
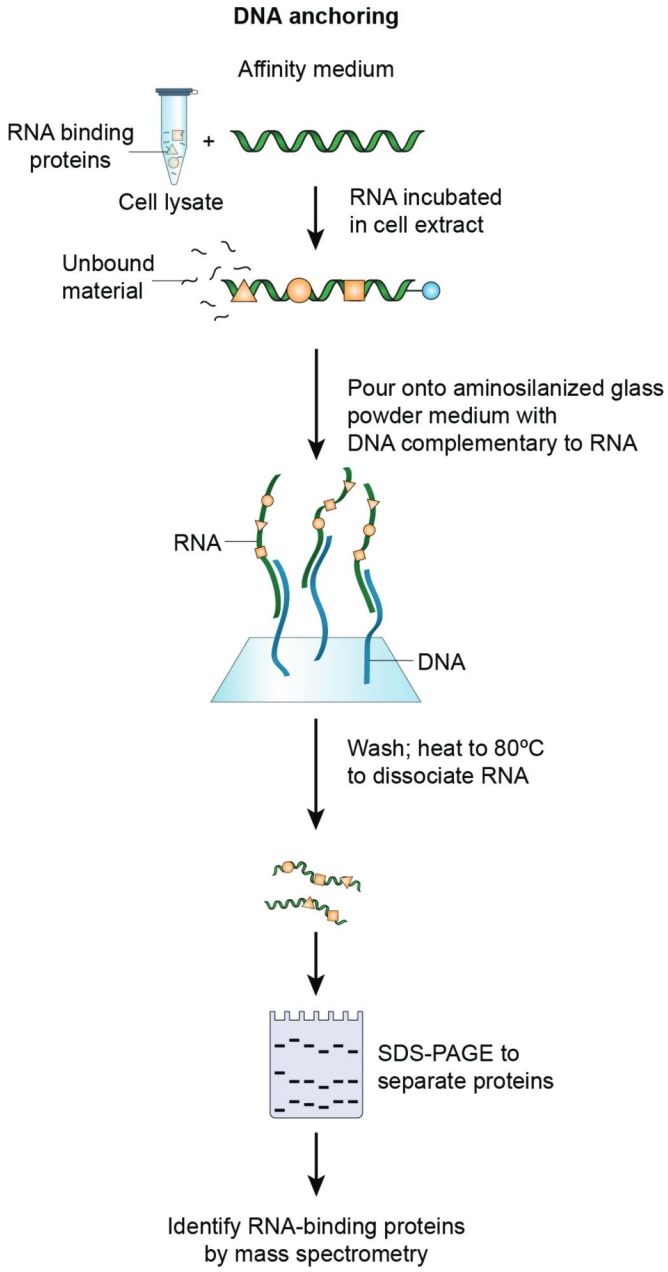


Fig. 6. The DNA anchoring approach for identifying RNA-binding proteins (RBPs). Once the aminosilanized plate is prepared, the ssDNA is ligated onto the plate. The RNA of interest is incubated with cell lysate to allow the formation of ribonucleoprotein complexes. The mixture is then added to the aminosilanized plate, followed by an incubation step to facilitate interaction between the RNA of interest and ssDNA. The unbound material is removed during a washing step, followed by heating of the samples to dissociate the RNA from the ssDNA. As with all of the other methods, the identification of RBPs is performed using mass spectrometry. [Colour online.]



ture and elution as described in Fig. 6 (Torres et al. 2018). An advantage to this approach is that the experiment can be carried out at lower temperatures (4 °C) to ensure RNA conformational changes remain in their native state. The disadvantage to DNA anchoring is that RBPs that interact more weakly with RNA are difficult to capture (Liu and Sun 2005).

Affinity medium capture

The affinity medium capture approach employs a solid surface to immobilize RBPs by creating an aminosilanized glass powder medium or plate, followed by anchoring a strand of ssDNA to that medium with a complementary strand that will base pair to the RNA being studied (Liu and Sun 2005). The RNA would be modified with a tail that is complementary to the ssDNA anchored to the medium. This allows the RNA to be suspended, allowing RBPs to bind to it. The elution is done by heating the entire complex to 80 °C for 5 min to denature the DNA–RNA strands. SDS–PAGE and mass spectrometry can be used to identify the proteins and EMSA or other binding studies to confirm RBP interactions (Liu and Sun 2005). Three cytoskeletal proteins were identified in this manner, revealing *trans*-acting factors that interact with RNA (Liu and Sun 2005).

One of the advantages of this approach is that the whole experiment can be performed at low temperatures, ensuring that the RNA conformations stay consistent under physiological or cooler temperatures. Further, the ssDNA glass-powder medium can be

used to anchor different RNAs that have been extended with the complementary strand, providing a platform for a lab performing multiple of these pull-downs. Additionally, according to the authors, the DNA-glass powder medium can be reused for multiple RNA pull-downs, implying at least three repeat applications of the same DNA-glass powder plate without compromise of function (Liu and Sun 2005). The elution step may be disruptive to the native RNA structure, however, and may need to be taken into consideration. One further disadvantage is that weakly-bound RBPs can be difficult to capture.

Discussion

Major strides have been made in RNA research by using RNA modifications to identify RBPs. Further research in this field has improved our understanding of the various biological processes that RNA molecules are involved in. However, much more work is required to understand the dynamics of RNA, and the various regulatory roles employed by RBPs. The application of chemically modified RNA is a powerful tool to answer critically important questions pertaining to RNA's involvement in gene silencing or editing, its involvement in unregulated cell proliferation in cancer, and how viruses use their RNA genome to hijack cellular machinery and propagate themselves (Hamilton et al. 2002; Jansson and Lund 2012; Hyodo and Okuno 2014).

Each technique described above has its strengths and challenges. It is important to keep in mind that each approach needs to be optimized for the system under study to identify important RBPs while reducing potential false-positives. The application of appropriate negative controls (e.g., scrambled nucleotide sequences, or the small molecule or an aptamer by themselves) is critical for the identification of RBPs. Typically, one should also employ more than one technique, where possible, to minimize false-positives. Such application of multiple techniques will provide common hits, which can subsequently be validated using biochemical and biophysical assays in terms of their direct interactions. Furthermore, it is essential to maintain RNA structure during modification to maximize the success of an experiment. As many of these techniques are relatively new discoveries, protocol optimizations and trial and error may lead to a pricey endeavor. Understanding the benefits and limitations of each of the previously described methods can allow researchers to pick a method that suits their experimental needs.

It is important to recognize that there are some inherent challenges associated with studying RNA and RNA–protein interactions. For example, RNase, which is present in abundance, is a very robust enzyme that even when autoclaved is not completely inactivated (Hirsch et al. 2002; Miyamoto et al. 2009). Therefore RNase-free conditions should be maintained throughout all of the experiments. Next are the challenges associated with the stability of RNA at higher temperatures. Depending on the secondary structure, temperature, and duration at elevated temperatures, RNA can be prone to degradation. For example, studies have demonstrated that at 25 °C, RNA has a degradation rate of 0.7–1.3 nt strand breaks per 1000 nt per century (inferred) when in RNase-free containers, whereas at 90 °C, RNA had 0.7–7.6 nt strand breaks per 1000 nt per 30 min (Fabre et al. 2014). While heating with subsequent slow cooling is often needed for proper RNA folding in-vitro, extended periods of time at these higher temperatures can compromise RNA quality and therefore downstream experimentation. Furthermore, when working with RNA, secondary structures and tertiary interactions are typically of utmost importance.

Some of the labelling techniques require temperature increases with the RNA sample, which, as a consequence, could change the native RNA conformation it adopts at physiologic temperatures (Fabre et al. 2014; Faoro and Ataide 2014). Further, the chemical modifications themselves may also influence or hinder the identification of some RBPs by mere attachment to a key RNA segment, and therefore need to be taken into consideration when designing experiments.

Overall, there are a multitude of RNA-labelling and anchoring approaches that may be used to identify RBPs. Understanding the advantages and limitations of these methods when interpreting experimental results is of utmost importance. We suggest that, at the very least, a second conformational approach is performed before concluding binding. Given the advances in our knowledge and understanding of the physiological roles of RNA and RBPs, the continued drive to study and understand these systems better will be complemented with one or more of the integral techniques described above.

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