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Biophysical studies of the translation initiation pathway using immobilised mRNA analogues

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Running title: Biophysical studies using immobilised RNAs

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Abstract

A growing number of biophysical techniques employ immobilised reactants for the quantitative study of macromolecular reactions. Examples of such approaches include surface plasmon resonance, atomic force microscopy, total internal fluorescence microscopy and others. Some of these methods have already been adapted for work with immobilised RNAs, thus making them available for the study of many reactions relevant to translation. Published examples include the study of kinetic parameters of protein:RNA interactions, and the effect of helicases on RNA secondary structure. The common denominator of all of these techniques is the necessity to immobilise RNA molecules in a functional state on solid supports. In this article, we describe a number of approaches by which such immobilisation can be achieved, followed by two specific examples for applications employing immobilised RNAs.

Introduction

Biochemical and biophysical experiments employing purified or *in vitro*-generated RNA molecules have a long history in translation-related research. Early insights into the workings of the translational apparatus were gained by studying the action of cell extracts or isolated ribosomes either on synthesized homopolymeric nucleic acids such as poly(U), or on easily obtainable, abundant natural transcripts such as globin mRNA. With the identification of the molecular components of the translational apparatus, characterisation of individual macromolecular interactions involving mRNAs became an additional focus of attention. The demonstration that RNA could be generated from DNA templates *in vitro* using RNA polymerases from bacteriophages (1, 2) greatly extended the range of RNAs available for use in these techniques.

Over recent years, a particular subset of biophysical techniques for the study of macromolecular reactions has developed in which at least one macromolecule is attached to a solid support. Several such techniques have been successfully adapted for work with immobilised RNAs, including surface plasmon resonance (SPR)-based approaches (3), atomic force microscopy (AFM) (4), total internal reflection fluorescence microscopy (TIRFM) (5), and laser-trap methods (6, 7). In addition, further biophysical methods are currently emerging (8, 9) that also rely on immobilisation of macromolecules, and which should eventually become useful for RNA-related work.

In the following chapter, we will first describe a selection of procedures for the generation and immobilisation of RNAs. We will then examine how two biophysical techniques (SPR and AFM) can be used to study these immobilised species.

Generation of RNAs

By far the most widely used mode of generating immobilised RNAs involves transcription of a DNA template *in vitro*, although, depending on the application, the purification of endogenous mRNAs or the purely chemical synthesis of oligomeric RNAs may be viable alternatives. In one of the procedures that can be used to facilitate immobilisation, biotin moieties can be randomly incorporated into *in vitro* transcripts simply by including biotinylated nucleotide derivatives in the transcription reaction. Although the biotins can then be efficiently used for immobilisation on streptavidin-containing surfaces, the random distribution of biotins throughout the RNA sequence will result in random orientations of the immobilised RNAs. Since it is generally desirable or even necessary to have more control over the position of the attachment site than this approach offers, RNAs are often synthesized in an unmodified state, and then modified for immobilisation via subsequent reaction steps.

Many suppliers now offer optimised kits for the *in vitro* transcription of DNA templates containing promoter sequences for either the T7 or SP6 bacteriophage RNA polymerases. Suitable templates for such reactions comprise single-stranded or double-stranded synthetic DNA oligomers, PCR products, and linearised plasmid DNA. Moreover, specialised kits are available for the introduction of eukaryotic mRNA end modifications like cap-structures. A typical *in vitro* transcription reaction involves the mixing of template DNA, ribonucleotides, purified RNA polymerase and required buffer components, incubation at 37ºC for 30 minutes to several hours, and finally removal of the template DNA by the addition of an RNAse-free DNAse preparation. The reader is referred to the optimised protocols normally accompanying the commercially available RNA polymerases.

An interesting alternative to *in vitro* transcription that can be used for the enzymatic generation of very short capped or uncapped RNA analogues has been developed by Matsuo *et al.* (10). In principle, their technique relies on a phage T7-derived enzyme called gene 4 primase, which produces short RNA primers beginning with the sequence pppAC on single-stranded DNA templates that contain the internal sequence GT. Further C residues can be appended to the reaction products if the GT recognition sequence is preceded by further Gs, i.e. for example the DNA template N_xGGGTN_y will result in the synthesis of a mixture of pppAC, pppACC and pppACCC. The longest RNA analogue that can be usefully synthesized with this method is $pppAC_5$, since yields drop dramatically with every additional residue. Although uses for gene 4 primase products are limited due to their shortness, this method is interesting because substituting ATP in the reaction with the dinucleotide cap-analogue m^7GpppA results in the quantitative production of capped oligoribonucleotides. Although the introduction of cap-structures into transcripts can also be achieved in standard T7 RNA polymerase *in vitro* transcription reactions, transcripts generated from templates containing internal Gs display heterogeneous 5'-ends because of the necessity of including both m^7GpppG (for generating the cap-structure) and GTP (for incorporation of guanosines during elongation) in the reaction mixture. In contrast, in the absence of ATP but the presence of m^7GpppA, gene 4 primase products are quantitatively capped, and are thus particularly useful in investigating cap-binding reactions. We are currently not aware of a commercial source of this enzyme, but protocols for its efficient purification have been published (11, 12). One alternative route to achieving 100% capping is to perform *in vitro* synthesis of an RNA that has only one G, ie. at the 5' end, although this approach can only be used in a restricted number of cases.

Modification and immobilisation of RNAs

Apart from the co-transcriptional incorporation of biotinylated nucleotides mentioned above, there are three principal approaches available to immobilise RNA sequences on a solid support (figure 1).

Oligo capture – a convenient way of immobilisation requiring relatively little experimental effort is the capture on chemically synthesized DNA oligonucleotides that include 5'- or 3'- biotin modifications. A 3'- biotinylated DNA oligomer can be stably attached to a Streptavidin-coated surface, and can be used to capture RNA molecules if its sequence is complementary to the 3'-sequence of the RNA to be captured (figure 1C). Conversely, a DNA oligomer that is 5'-biotinylated can be used to capture a complementary RNA via its 5'-terminal sequence. The strength of immobilisation with this approach is dependent on the strength of the DNA:RNA duplex being formed, i.e. essentially on the length of the complementary region of RNA and DNA oligomer. An advantage of this approach is that surfaces can often be easily regenerated by dissolving the DNA:RNA duplex. However, results published using oligo capture onto a BIAcore chip show that the captured RNA is released with significant rates even under normal buffer conditions and in the absence of any external force (see e.g. ref. (13)). Therefore, although this method of immobilisation is very convenient, the stability of binding needs to be evaluated for every individual application and experimental setup.

End labelling with biotinylated residues – one method for the targeted introduction of biotinylated residues at the 3'-end of an RNA molecule relies on the ability of certain enzymes to append nucleotides to existing RNA sequences. One published method employs poly(A) polymerase to incorporate biotinylated adenine moieties at the 3'-end of a transcript previously generated by standard *in vitro* transcription (14). In

contrast to the oligo-capture method, the biotin moieties are covalently attached to the RNA, and the strength of the bond to the solid support thus corresponds to the strength of the biotin-Streptavidin bond, i.e. it is quasi-covalent. The protocol for this procedure is given in detail below (protocol 1).

Chemical modification – an alternative strategy to the widely-used co-transcriptional introduction of biotins into transcripts is the introduction of chemical groups into the RNA that allow for the establishment of direct, covalent bonds to the surface. Depending on the application, different surface chemistries may be available, although NHS (*N*-hydroxysuccinimide) -ester based chemistries for the coupling of amines have been most widely adopted and are available for most applications. Two published chemical modifications, the introduction of aldehyde groups at the RNA 3'-end and the introduction of a thiol-group at the 5'-end, are described in detail in protocols 2 and 3. These modifications can be used for covalent binding to NHS – derivatised surfaces by using bi-active compounds containing an amine-group for direct reaction with the surface, and a second group for reaction with the modified RNA ends (e.g. cystamine for coupling of the sulphur moiety; hydrazine for coupling of the aldehyde).

Using immobilised RNAs in the BIAcore system

In the BIAcore system, published studies employing immobilised RNAs now range from the study of individual protein:RNA and RNA:RNA interactions (15, 16) to the formation of complete *E. coli* initiation complexes (17). A problem for the study of RNA:protein interactions is that, on surfaces containing immobilised RNAs, the high density of phosphate moieties creates a relatively high net negative charge. This

charge attracts proteins with an isoelectric point above the relevant buffer conditions, and can lead to strong, non-specific ionic interactions. In addition, some non-specific binding can even be observed if the binding protein has a low isoelectric point, but contains localised patches of positive charge. We have found that the inclusion of tRNAs in the eluent buffer to a final concentration of 20 mg/l can greatly reduce non-specific binding of the cap-binding protein eIF4E to uncapped RNAs, while it has no effect on the specific association of this protein with capped RNAs (18). This strategy may be generally applicable when studying binding to specific RNA elements, where sequence-independent binding can be out-competed by the soluble tRNA population. While immobilised RNAs are thus well suited for studying binding of proteins to specific sequence elements, sequence-independent RNA binding is best studied using immobilised proteins and soluble RNAs.

We have previously used the principle of studying protein:RNA interactions via immobilised RNAs in the BIAcore for investigating the interaction between the cap-binding protein eIF4E and small, capped mRNA analogues that contained single biotin-moieties near their 3'-ends (figure 2A). Although biotin was incorporated co-transcriptionally via biotin-UTP in this case, site-directed labelling was achieved by using a chemically synthesized DNA oligomer as template that introduced only a single U residue near the 3' end of the transcribed sequence. Extravidin (Sigma E2511), a Streptavidin derivative with favourable non-specific binding characteristics, was immobilised on CM5 sensor chips via a standard amine coupling kit (BIAcore BR 1000-50), and the biotinylated RNA was then captured on the immobilised extravidin (Figure 2B). As a control, a second sensor cell was loaded in the same way with a non-capped RNA, to which eIF4E binds with greatly reduced affinity compared to the capped version.

Simultaneous injection of eIF4E over the two surfaces results in a binding signal resulting from non-specific binding to the RNA in the control cell, and a binding signal resulting from non-specific binding plus specific binding to the cap-structure in the control cell (schematically shown in figure 2C). A pure signal for cap-specific binding can then be generated by subtracting the non-specific signal from that obtained with the capped RNAs.

The extraction of thermodynamic and kinetic data from the curves thus obtained is usually performed in one of two ways. Most frequently, the curve fitting software that is part of the BIAcore package is used to fit the experimentally obtained curves to theoretical binding models. This assumes some prior knowledge of the actual binding model of the interaction, which is usually either a simple Langmuir interaction of the form A + B <-> AB, where A and B are the interacting components and AB is the complex, or a two-step interaction of the form A + B <-> AB* <-> AB, where AB* is an intermediate, unstable complex that can either rapidly decay into its components, or undergo a conformational rearrangement to form the final, stable complex AB. Some more complex binding models can also arise from parallel binding reactions caused by impurities or heterogeneities in either of the two binding partners. While the BIAcore curve fitting software provides pre-configured models for all of these cases, it is worth noting that the more complicated binding models contain a greater number of free parameters than the Langmuir model, and will thus almost always result in apparently better fits. In order to decide whether the application of more complicated models is appropriate, it is therefore essential to test experimentally whether such models are meaningful, for example by providing evidence for a conformational rearrangement during the interaction that could give rise to a two-step binding mechanism.

Curve fitting will produce detailed kinetic data including values for the on- and off-rates of the interaction, but this procedure is limited by the number of data points that are generated before an equilibrium is reached. As can be seen in the example of the eIF4E:cap interaction described below, very fast off-rates reach an equilibrium within seconds or less, and in this case the small number of data prior to reaching a plateau in the SPR signal does not permit meaningful curve fitting to be performed. However, in this case equilibrium binding levels (represented by the plateau in the binding curve reached during the injection of the binding partner) can be easily analysed, and a simple plot of equilibrium binding levels against concentration of the binding partner allows at least the analysis of relevant equilibrium binding constants. In BIAcore experiments, the latter are equivalent to the concentration of injected binding partner at which half-maximal equilibrium binding levels are obtained.

Some typical data obtained with the cap-binding protein eIF4E2 from *S. pombe* (19) are shown in figure 2D. The resulting sensorgrams are indicative of a reaction where eIF4E cycles extremely fast on and off the cap-structures. For the reasons explained above, the on- and off rates of this interaction are too fast to be directly determined by curve fitting. However, the equilibrium affinity could easily be determined by plotting the equilibrium binding levels against the concentration of the injected protein (19). The rapid binding/ release cycle for the yeast eIF4E:cap interaction was later confirmed in stopped-flow experiments, which revealed on-rates of 10^8-10^9 M^{-1} s^{-1} for the interaction with m^7GTP, close to the apparent diffusion limit for this interaction (TvdH, unpublished data). Similar association rates were also obtained for the human protein (20). In summary, these and other published data show that immobilised RNAs can be a useful tool for studying a variety of protein:RNA interactions.

Using immobilised RNAs for atomic force microscopy

In atomic force microscopy (AFM) experiments, immobilised RNAs can be used to probe secondary structure elements as well as the effect that RNA binding proteins have on the stability of these structural features (figures 3 and 4). Essentially, it is the force required to pull apart secondary structure elements in an RNA that is measured with this particular technique. As an RNA suspended between a surface and an AFM cantilever tip is stretched by slow movement of the tip, the force applied to the latter increases with the distance between tip and surface (figure 4A). However, the opening of secondary structure elements will increase in sudden drops of force applied to the tip (see the transition from step iii to step vi in figure 4A), resulting in so-called discontinuity features in the force-distance curves. Analysis of these discontinuity features allows for the interpretation of parameters relevant to the stability of the secondary structures introduce into the immobilised RNA.

We have recently described a series of such experiments designed to probe the effect of RNA helicase activity on artificial hairpin structures introduced into the yeast *GCN4* mRNA leader (4). Suitable RNAs that can be attached to a gold-coated glass slide via a 5` thiol modification, and that have a biotinylated 3` poly-A tail allowing pick-up by a streptavidin coated AFM tip (as shown in Figure 3), can be synthesised by sequential modification of a transcript with the procedures described in protocols 3 and 1.

For the best results, the RNA should be at least 600 nt long and contain a maximum of two stable stem-loops. The *GCN4* leader sequence from yeast is an example of a suitable, relatively 'structureless' control construct into which specific stem-loop sequences can be introduced. The RNA construct can be transcribed from this template using individually prepared transcription components; however, we

have found that very good yields can be achieved using an RNAmaxx transcription kit (Stratagene). A high initial yield of 100 µg is required here because only 10 % of the transcript may remain after the two subsequent modification steps. In order to minimise RNA degradation, gloves should be worn at all times, and DEPC-treated water containing RNase inhibitors should be used in buffers where possible. Also, the sample should be kept on ice between steps and stored at -80°C overnight.

Coating of AFM tips with streptavidin – Si_3N_4 NPS AFM tips (Veeco DNPS-20) were modified by soaking overnight in 50 µl BSA-biotin (1mg/ml (Sigma A8549-10MG)) at 37°C. The tips were then washed with 50 µl DEPC-treated H_2O and fixed in 1% glutaraldehyde (Sigma G7651) for 30 secs before being washed again in 50 µl DEPC-treated H_2O. Immediately before an experiment, 50 µl recombinant streptavidin (1mg/ml, Roche Diagnostics 11721666001) were added and allowed to bind for 5 mins. The tips were then washed with 50 µl DEPC treated H_2O.

Binding of the RNA construct to a gold-coated AFM slide – in order to allow primarily single molecules to be picked up, the RNA constructs are best added to the gold surface at a concentration of approximately 500 pM, although this may vary according to conditions. Our purified Th-RNA-Biotin constructs were routinely maintained at a higher concentration of 2.7 nM for long term storage. For application to the gold surface, we diluted 1µl of this stock into 50 µl of 150 mM NaCl, 1xBSA, 10 mM TrisHcl, 10 mM $MgCl_2$, 1 U/µl RNasin in DEPC-H_2O. Immediately before experimentation, 10 µl of the diluted RNA were spread evenly over a 11 mm x 11 mm gold-coated slide (Gold-Arandee^{TM}, www.Arrandee.com) and left at room temperature for 20 mins. The slide was then washed three times with 200 µl of 'RNA pulling buffer' (15 0mM NaCl, 10 mM TrisHcl, 10 mM $MgCl_2$, 1 U/µl RNasin) by tilting the slide using tweezers and allowing capillary action to draw the solution off

the slide. The slide was then quickly placed on the AFM stage (via a mounting disk coated in adhesive) and 50 µl of RNA pulling buffer was dropped onto it. The BSA in the RNA binding buffer is sufficient to reduce non-specific tip-surface interactions, which are in any case usually of minor importance since the recorded events (the opening of a stem-loop) occur only after the backbone of the RNA is stretched out and the tip is physically removed from the surface.

Collecting AFM force spectroscopy data – the following describes the procedure for obtaining RNA force curves using a Digital Instruments AFM (Veeco), with a multimode head, PF scanner, Nanoscope IIIa and Picoforce controllers with an extender module and Nanoscope software. AFM RNA force spectroscopy was performed using the liquid cell and a short (100 µm) V-shaped silicon nitride AFM cantilever with a spring constant of approximately 0.02 N/m, as these produce the least noise of the tips tested. The tip was coated with streptavidin as described above. Before placing the liquid cell into the AFM head, a 2 µl drop of RNA pulling buffer was placed next to the tip. The buffer drop is then drawn underneath the tip by capillary forces, which prevents the formation of air bubbles when the tip is lowered onto the sample buffer.

The laser was then aligned on the back of the cantilever and the spring constant of the cantilever estimated using the thermal tuning method while the tip was in the sample buffer but still greater than 2 µm from the surface. The tip was manually lowered close to the surface, and the software was used to engage the tip in contact mode with a scan size of less than 1 nm and a set point of 1.5 V. Switching to picoforce mode allowed force curves to be generated. A single force curve produced with the trigger force disabled and a ramp length of 1 µm should show a constant compliance region

as the tip pushes into the surface. This region was used to determine the cantilever deflection sensitivity.

In order to obtain RNA force curves, continuous cycles of approach and retract with a trigger force of 200 pN, retract velocity of 1 μm/s, a surface delay of 400 ms and a ramp length of just above the contour length of the RNA construct were set up. The tip was moved around the surface in the x and y directions in 100 nm increments until an area was found in which many force-distance curves with long adhesion lengths before pull-off could be measured. Once in this area, the ramp length was reduced to less than 200 pN. Force curves in which both the approach and retract curves bend (rather than the retract curve only) started to appear at this ramp length. The approach curve also bends because the RNA remains attached to the tip at the end of the retract cycle. Once it was evident that the RNA had been picked up stably in this way, the separation from the surface was increased manually using the picoangler, until the contour length of the RNA construct was reached.

Using this method, up to one hundred consecutive full length, reversible, RNA stretching force curves per RNA molecule should be obtainable. Once the RNA is pulled off the tip, and if it does not re-attach within a couple of minutes of pulling, then the tip should be moved to a new position on the slide and the process should be repeated. We found that the RNA preparation can last up to 4 hours before RNA force-distance curves can no longer be generated.

In order to ensure the authenticity of the data produced in these experiments, a series of controls need to be performed. In particular, controls with an unmodified RNA transcript, and with thiolated but not biotinylated RNA, and biotinylated but not thiolated RNA, should show a greatly reduced occurrence of long adhesion length force curves compared with the complete Thiol-RNA-biotin constructs.

At a concentration of 540 pM, control RNA constructs with no specific secondary structure should produce force curves with no discontinuity features within the last 50 nm of the pull in 98 % of curves. Strong (>50 kcal/mol) GC-rich stem loops placed within the construct should introduce clear discontinuities (like those shown in Figure 4) in >70 % of full length RNA curves. Weaker structures (50 kcal/mol) show discontinuities that are less visible above background noise and can be identified in less than 50 % of curves.

Since the AFM operates at a higher loading rate than is used by other force spectroscopy techniques (such as laser tweezers) and than is assumed by theoretical predictions, measurements should always be taken at different loading rates within the range of the AFM. The modal force should increase linearly as the logarithm of the loading rate is increased. In some cases this allows extrapolation back to the lower loading rates used by other methods in order to obtain comparable force estimates. However, if the unfolding event follows a different pathway at the higher range of loading rates then the extrapolation may meet the x-axis before the lower loading rates are reached. An approximate value at lower loading rate may still be obtainable however by plotting the mean force of stem-loop opening directly against loading rate and using curve fitting software to extrapolate backwards.

Analysis of AFM force spectroscopy data – as the process of selecting RNA curves and then identifying secondary structure features on these curves is potentially highly subjective, we have introduced a disciplined regime designed to help in deciding which curves to include. As a test for subjectivity, a series of 'blind' experiments were performed, in which sets of force curves generated with secondary-structure free and stem-loop containing RNAs were mixed and subsequently analysed. The two

types of construct could be reliably distinguished by this analysis, hence proving that any preconceptions about the data do not bias the analysis.

The amount of secondary structure detected by AFM force spectroscopy can be represented in terms of the percentage of force curves containing a discontinuity feature and by the opening force distribution of these features. For reliable analysis, opening forces from many hundreds of curves need to be collected into histograms. When collecting statistics, Th-RNA-biotin force curves are taken to be those reversible curves that display the distinctive initial gradual slope, that reach an adhesion length of at least 250 nm, and a force of at least 180 pN. The stem-loop opening features are recorded when only a single discontinuity is seen in the last 50 nm of the pull. Only curves where the region between 50 nm from the start and 50 nm from the end of the pull is smooth are counted in the statistical analysis (in practice this includes the vast majority of curves under our experimental conditions). Curves with a single discontinuity force larger than 250 pN are counted as extraordinarily large forces due to potential stabilisation of the stem-loop by protein binding and so should not be included in the opening force statistics.

Investigating helicase activity using AFM RNA force spectroscopy – RNA helicases can be added to the described AFM RNA force spectroscopy system in order to measure their effect on RNA secondary structure. AFM RNA force spectroscopy was at first set up to take measurements on RNA alone, to ensure that the tip and working conditions were suitable for producing good RNA force curves. The AFM slide was then removed and washed with 200 µl RNA pulling buffer. Then, 50 µl of RNA pulling buffer containing 1 mM ATP and the RNA helicase were added to the surface. Generation of RNA force curves was then started as quickly as possible. We found that pumping RNA helicase directly into the liquid cell is not advisable as this may

cause removal of RNA from the gold slide and does not allow the concentration of helicase supplied in the solution to be accurately controlled. Moreover, it will not allow instant measurements upon the addition of helicase because pumping in solution invariably detaches RNA from the AFM tip and temporarily destabilises the system. A potential solution to this problem would be to use a helicase buffer containing ATP that is trapped in a photosensitive cage and that could be released upon exposure to UV light (from a flash lamp) in order to activate the helicase without disturbing the RNA attachment.

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Figure legends

Figure 1. Methods for the generation of RNAs for immobilisation. **A,** biotinylated nucleotides such as Biotin-UTP or Biotin-ATP can be incorporated into the transcript by including the modified nucleotides in the transcription reaction. The resulting transcript is randomly labelled , and hence the orientation and attachment points of the immobilised RNA will also be of a random nature (giving rise to heterogeneity). **B,** biotinylated nucleotides can be appended to *in vitro* transcripts via enzymatic activities (i.e. poly(A) polymerase for the introduction of biotin-ATP at the 3'-end). **C,** transcripts can be captured via complementary, chemically synthesized DNA oligonucleotides that contain 5'- or 3'- biotin modifications. **D,** transcript ends can be chemically modified in order to introduce chemical groups (here an aldehyde group) that are suitable for covalent attachment to reactive groups contained on solid surfaces.

Figure 2. Use of immobilised RNA for measuring the eIF4E:cap structure interaction, as an example of the usefulness of the SPR techniques. **A,** primary structure of the *in vitro* transcript used for the experiments. Biotin-UTP was inserted co-transcriptionally. **B,** Experimental setup for measuring the eIF4E:cap association. Extravidin (a modified Streptavidin derivative) was covalently immobilised on a sensorchip surface by standard amine coupling. The biotinylated RNA was then captured on the Extravidin, and eIF4E was injected over the RNA-containing surface. **C,** principle of control cell substraction. eIF4E will associate with surfaces containing uncapped RNAs via unspecific interactions, and with surfaces containing capped RNAs via a combination of specific and non-specific interactions. Subtraction of the control sensorgram on the left from the sensorgram on the right yields a pure sensorgram that reflects only the specific eIF4E:cap interaction. **D,** real-time, control-subtracted sensorgrams from the injection of eIF4E over immobilised, capped RNAs at four different concentrations.

Figure 3. A summary of the method used for suspending an RNA molecule between the AFM tip and a gold-coated microscope slide. The RNA molecule was attached to the gold surface via a thiol modification at the 5' end. The 3' end was modified by the

incorporation of Biotin-ATP residues, which allowed picking up of this end with a streptavidin-coated cantilever tip.

Figure 4.

A, A theoretical force-extension curve for structured-RNA stretching carried out by the AFM. The curve is annotated with cartoons showing the state of the RNA molecule and AFM cantilever at different extensions. (i) RNA is uncoiled off the gold surface causing an entropic force increase; (ii) Very weak secondary structure interactions are removed; (iii) Enthalpic forces increase as the molecule is pulled taut and force becomes high enough for the hydrogen bonds in the strong specific stem-loop to break; (iv) Force temporarily decreases as the slack released from stem-loop opening is pulled out; (v) Force increases further once the RNA is pulled taut once more. **B,** A theoretical retraction force curve for the stretching of the *GCS4* L1-RNA transcript (with a 65nt Poly(A) tail). This trace is adapted from data generated using the online 'RNA pulling server' at http://bioserv.mps.ohio-state.edu/rna (21). The model assumes a temperature of 37°C, 1M NaCl, and a nucleotide length of 0.334nm. The predicted ~40pN GC-rich stem-loop opening feature is labelled with an arrow. **C**, Example of an AFM force-curve representing stretching of a *GCN4* RNA molecule containing the GC-rich (25 base pair) stem-loop (inset). The discontinuity feature resulting from stem-loop opening is indicated by a small arrow. The approach curve (Bottom Curve) runs from right to left and the retract curve (Top Curve) from left to right.

Protocol 1 – 3'-end biotinylation of a transcript using poly(A) polymerase.

Starting point for this protocol is a T7 RNA polymerase transcript that has been DNAse 1 treated and purified using phenol extraction and ethanol precipitation. The volumes and amounts given are optimal for biotinylation of the product of a 25 µl reaction using the RNAmaxx kit (Strategene #200339).

Additional Reagents: Poly-A polymerase with 5x buffer (Amersham Biosciences E74225Y), RNAse inhibitors (e.g. Promega N2111) 1 mM Biotin-17-ATP (Enzo labs 42817) 10 mM ATP (**Sigma A1852**), probequant G-50 microcolumns (Amersham Biosciences 28-9034-08). Also required is DEPC-treated water.

1. Resuspend the precipitated RNA in 17 µl of DEPC-treated water. Add 1 µl RNAse inhibitor, 6 µl 5x Polymerase buffer, 4 µl Biotin-17-ATP, 1 µl ATP and 1 µl of the polymerase and mix.
2. Incubate at 37º for 30 minutes.
3. For removal of the unincorporated nucleotides, equilibrate a G-50 microcolumn by addng 50 µl DEPC-treated water and centrifuging at 2000 rpm for two minutes. Repeat this step twice.
4. Add the polyadenylation reaction to the column, stand at room temperature for 30 seconds, then spin at 2000 rpm for 2 minutes.
5. Analyse a small sample of the eluate on an agarose or polyacrylamide gel suitable for the size of the original RNA. Successful adenylation can be observed by an apparent increase in molecular weight of the transcript, as well as a smeared appearance of the band. If necessary, biotinylation can also be demonstrated using an anti-biotin antibody.

Protocol 2 – Introduction of aldehyde groups by oxidation of the mRNA 3'-end.

Starting point for this protocol is a T7 RNA polymerase transcript that has been DNAse 1 treated and purified using phenol extraction and ethanol precipitation. The volumes and amounts given are suitable for biotinylation of the product of a 25 µl reaction using the RNAmaxx kit (Strategene #200339).

Additional Reagents: 0.1 M Sodium acetate pH 5.1, sodium meta-periodate ($NaIO_4$, Sigma S1878), NAP 5 column (Pharmacia), DEPC-treated water.

1. Dissolve the pelleted RNA in 225 µl of the sodium acetate solution.
2. Freshly dissolve 20 mg of the meta-periodate in 1 ml of water. Add 25 µl of this stock to the dissolved RNA (to give ~10 mM final concentration of the meta-periodate).
3. Incubate the mixture for 1 hour in the dark.
4. Ethanol precipitate the RNA, redissolve the pellet in 250 µl of water.
5. Pass the redissolved RNA through a NAP5 column as per the manufacturers instruction to remove all traces of the oxidising agent.

This procedure essentially destroys the ribose moiety of the 3'-terminal nucleotide by cleaving the ribose ring and introducing two aldehyde groups at the C2 and C3 carbons. Procedures for covalently binding oxidised RNAs e.g. to BIAcore chips have been described (17), and can be performed using standard coupling kits available from this company.

Protocol 3 – Thiolation at the mRNA 5'-end using polynucleotide kinase.

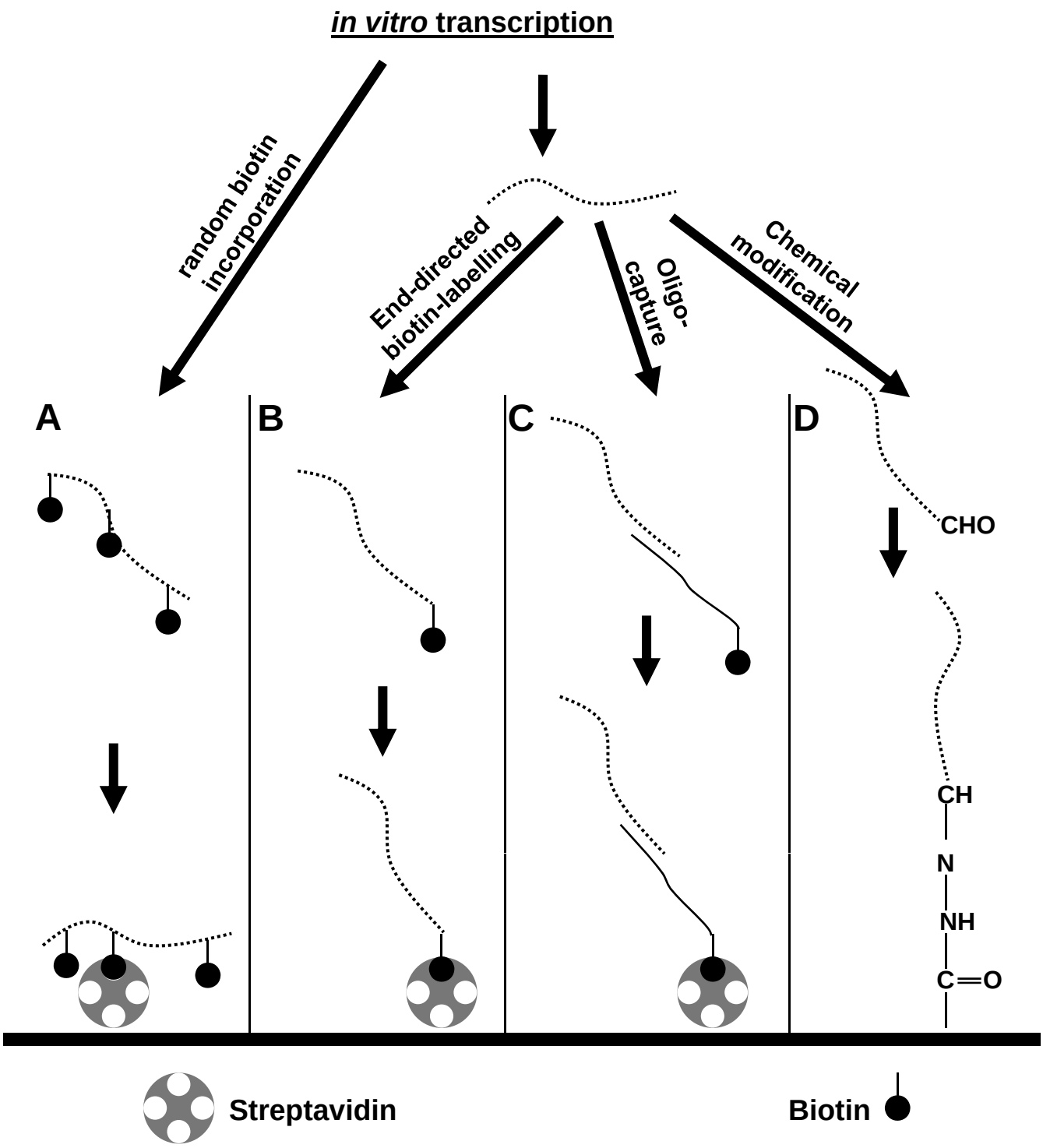
Starting point for this protocol is a T7 RNA polymerase transcript that has been DNAse 1 treated and purified using phenol extraction. For complete removal of unincorporated nucleotides, the RNA pellet should be dissolved in water, passed through a desalting column (e.g. Amersham G-50 microcolumns) and then precipitated again with ethanol. The volumes and amounts given are optimal for modification of the product of a 25 µl reaction using the RNAmaxx kit (Strategene #200339).

Additional Reagents: 5' end labelling kit (Vector Labs MB-9001) including shrimp alkaline phosphatase, ATPγS, reaction buffer and polynucleotide kinase; RNAse inhibitors (e.g. Promega N2111), DEPC-treated water.

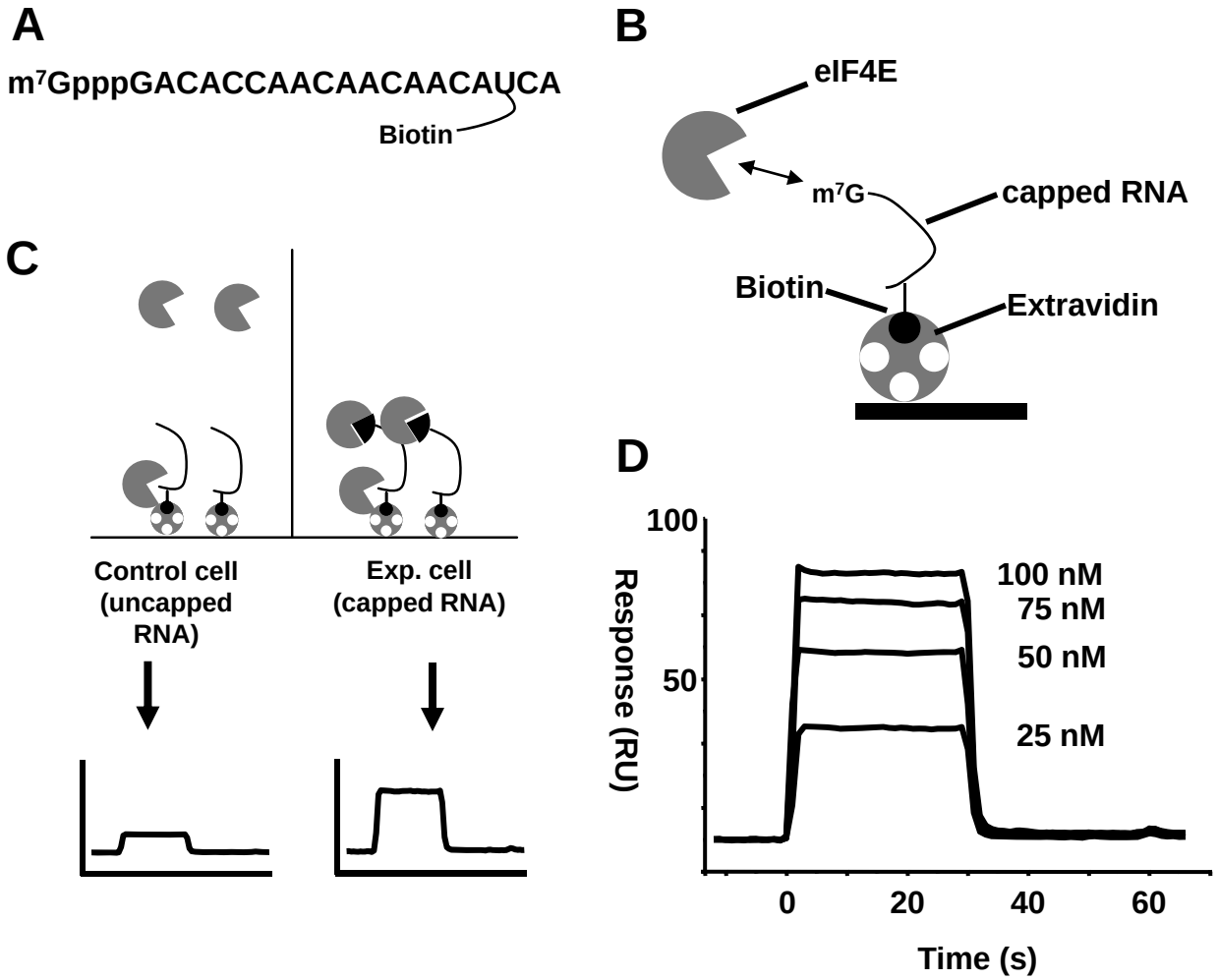
1. Resuspend the precipitated RNA in 7 µl of DEPC-treated water.
2. Add 1µl universal reacton buffer, 1µl RNAse inhibitors and 1 µl of the shrimp alkaline phosphatase. Incubate at 37ºC for 30 minutes.
3. Add 2 µl of universal reaction buffer, 1 µl ATPγS, 6 µl of DEPC-treated water and 2 µl polynucleotide kinase. Incubate at 37ºC for 30 minutes.
4. Make up the volume to 100 µl by adding 79 µl of DEPC-treated water. Purify the labelled RNA by phenol extraction and ethanol precipitation.

Thiol-incorporation on intact RNA can be detected by taking a 2µl aliquot from the sample at the end of step 6 and incubating with 20µg Biotin-PEG_3-Maleimide (BP_3M) in a final volume of 40µl DEPC-H_2O at 65°C for 30mins, running on a 12cm 2.2% denaturing formaldehyde gel for 2hrs at 100V, 400mA and performing a Northern blot onto a nylon membrane. The membrane is then blocked by soaking for 30mins in 20ml blocking solution (1% casein, 1xTBS (10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20)) and soaked for 4hrs in 20ml of a solution containing 0.5%

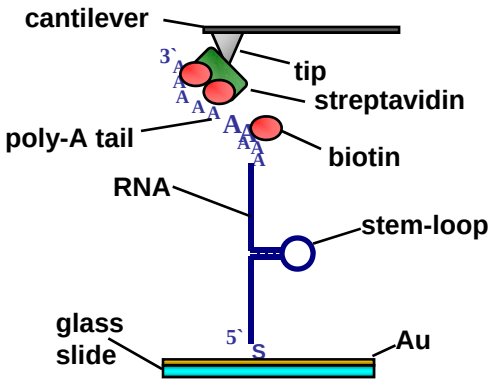
casein, 1XTBS, 5µg streptavidin-Alkaline phosphatase, before being washed 3 times in 1XTBS. The blot is then developed by soaking in 30ml developing solution (50mM $NaHCO_3$, 5mM MgCl, 70µg NBI, 16µg BCIP) for 10mins. Brown bands on the gel indicated AP activity hence indirectly showing the presence of the thiol. Alternatively, BP_3M-Th-RNA can be run on a G-50 column and then spotted directly onto a nylon membrane that is probed and developed as above. Comparing the intensity of the brown spot with that produced using a commercially available Th-RNA oligo (assumed to have 100% Th-incorporation) allows the percentage of Th-incorporated to be determined. Thiolation of RNA according to the profile described here typically gives a yield of 10-15%.



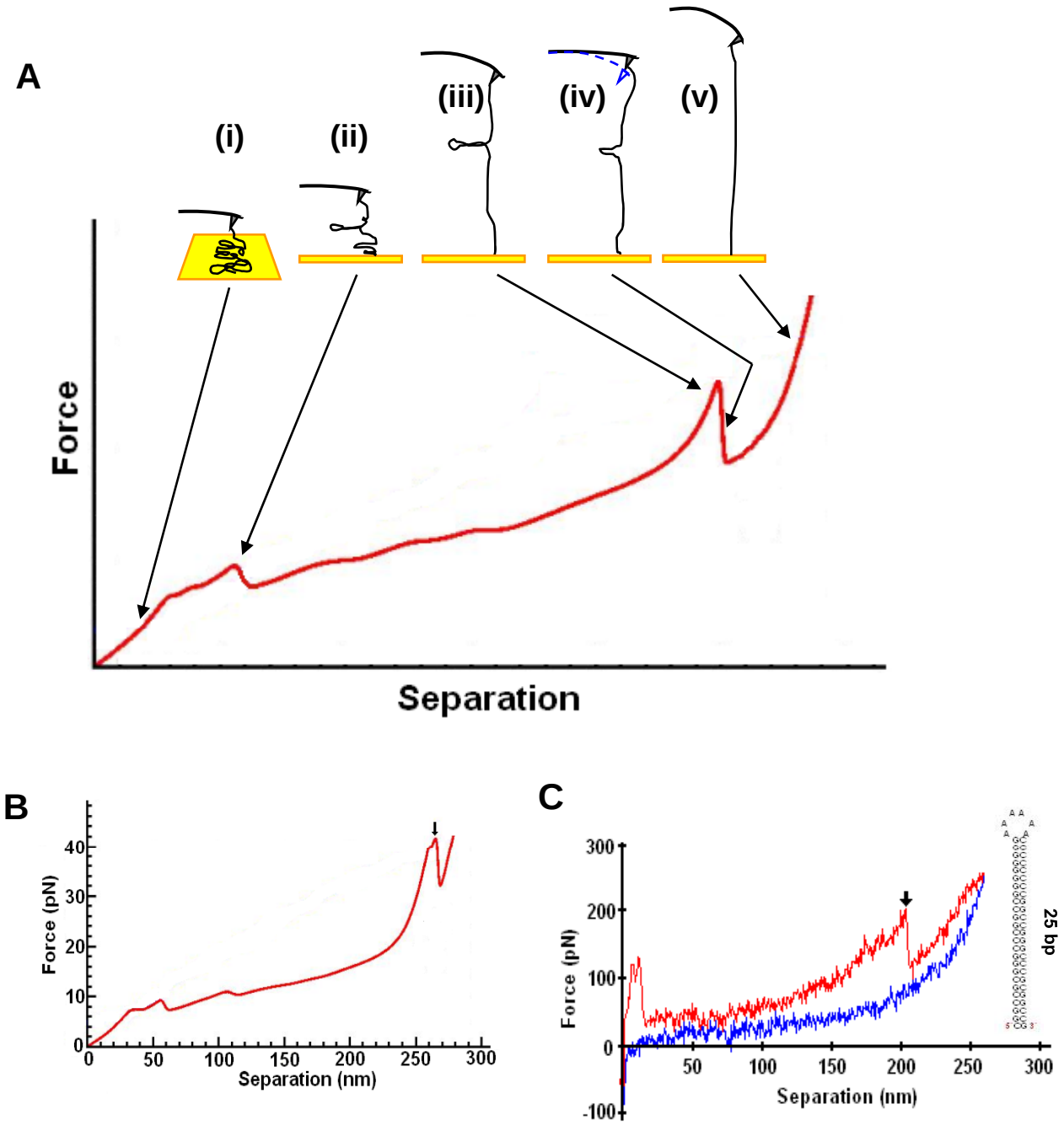
McCarthy et al. 2007 Figure 1



McCarthy et al. 2007 Figure 2



McCarthy et al. 2007 Figure 3



McCarthy et al. 2007 Figure 4