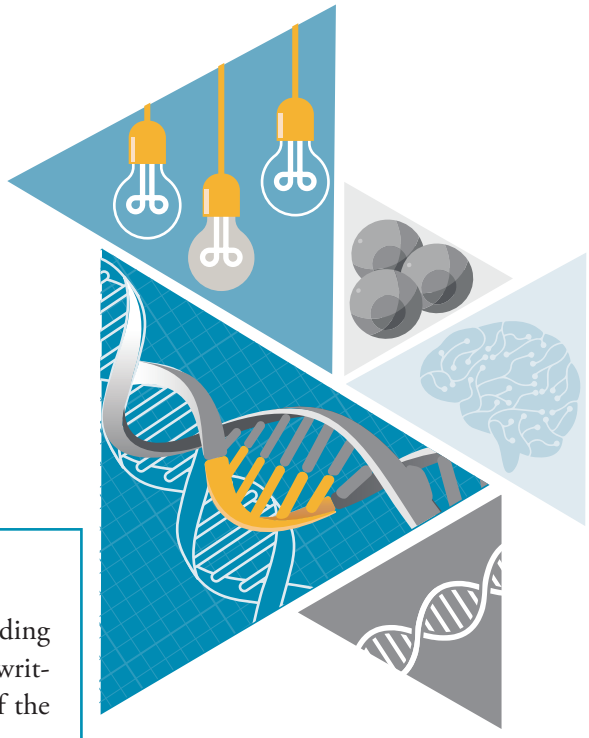
CELL & GENE THERAPY INSIGHTS



Innovation Insights

-

CORRIGENDUM

In the version of this article initially published, the stated binding capacity of the CIMmultus Oligo dT column was incorrectly written as 0.18mg$_{mRNA}$.mL$_{support}$-1. The correct binding capacity of the CIMmultus Oligo dT column is more than 20× higher. This error has been corrected in the article below as of January 9 2023.

REVIEW

Purification of therapeutic & prophylactic mRNA by affinity chromatography

Julian Grinsted, John Liddell, Emir Bouleghlimat, Ka Yan Kwok, Georgia Taylor, Marco PC Marques & Daniel G Bracewell

In vitro transcribed mRNA is an emerging therapeutic and prophylactic modality with the potential to transform medicine. The drug platform features exceptionally rapid development and versatility of manufacturing processes. Despite the prompt advancement of mRNA from trials to market, purification challenges remain. The cell-free synthesis of mRNA is responsible for the generation of product and process-related impurities, creating the potential for immunogenic effects and decreased translatability into the clinic. Affinity chromatography presents itself as an effective primary capture step for the isolation of functional transcripts from product and some process related impurities. Developing platform processes for the affinity purification of mRNA is hindered by the varying strand lengths of non-amplifying, self-amplifying, and trans-amplifying constructs, with disparities in capacity being observed. Ligand chemistries may contribute to non-specific binding events which remain challenging to characterise. Improved elution and wash conditions may be pursued through novel ligand chemistries, enhanced density and spacing. Regardless of the size or application of the product, the impurities generated by *in vitro* transcription represent a significant obstacle to the safe administration and long-term storage of mRNA. Affinity chromatography is a valuable tool in overcoming these challenges, with current commercially available products relying

heavily on oligo deoxythymidine ligand chemistries. Whilst affinity chromatography is highly valuable in the purification of mRNA, the inability to separate key secondary structures such as double-stranded RNA means it remains to be seen if this technology will adopt the same position as protein A does in mAb manufacture.

Cell & Gene Therapy Insights 2022; 8(2), 341–355

DOI: 10.18609/cgti.2022.049

The COVID-19 pandemic has shown a rapid response from vaccine companies, manufacturing and delivering mRNA vaccines in record time. Unlike traditional vaccines which rely on complex and inflexible manufacturing processes, mRNA vaccines use the same vaccine backbone for multiple targets with only the expression of the gene of interest, allowing standardised manufacturing with reduced footprints (Table 1). This will enable the manufacturing of different mRNA vaccines using the same production platform. Furthermore, the facilities and manufacturing techniques can be applied to a variety of different products with varying applications, such as vaccines against infectious diseases, cancer immunotherapeutics and protein replacement therapies [1] (Figure 1). However, the global demand for COVID-19 vaccines has placed strain upon global manufacturing and supply chain problems are arising [2]. Therefore, new, or optimised processes are necessary to cope with increased demands of these vaccines. In particular, the purification of mRNA, where the *in vitro* production of mRNA has given rise to unique purification challenges such as low capacity and the removal of immunogenic impurities [3,4].

In 1990, mRNA molecules were successfully synthesised *in vitro* using free enzymes and expressed in mice to produce three proteins: chloramphenicol acetyltransferase, β-galactosidase, and luciferase [5]. Despite this initial success, DNA-based therapeutics were still preferred as mRNA is easily degraded by RNases present in cells and presents overall lower stability at ambient temperatures compared to DNA [6]. Inherently, mRNA based vaccines present safety advantages compared to DNA vaccines: the mRNA cannot interact with the cell genome; the mRNA consists solely of the elements needed for expression of the encoded protein; the mRNA decays within a couple of days and is non-replicative [7]; *in vivo* transfection rates are high due to the fact mRNA only has to cross the cell plasma membrane [8]; For these reasons multiple mRNA vaccine candidates such as the Pfizer-BioNTech BNT162b2 and the ModernaTX mRNA-1273 mRNA SARS-CoV-2

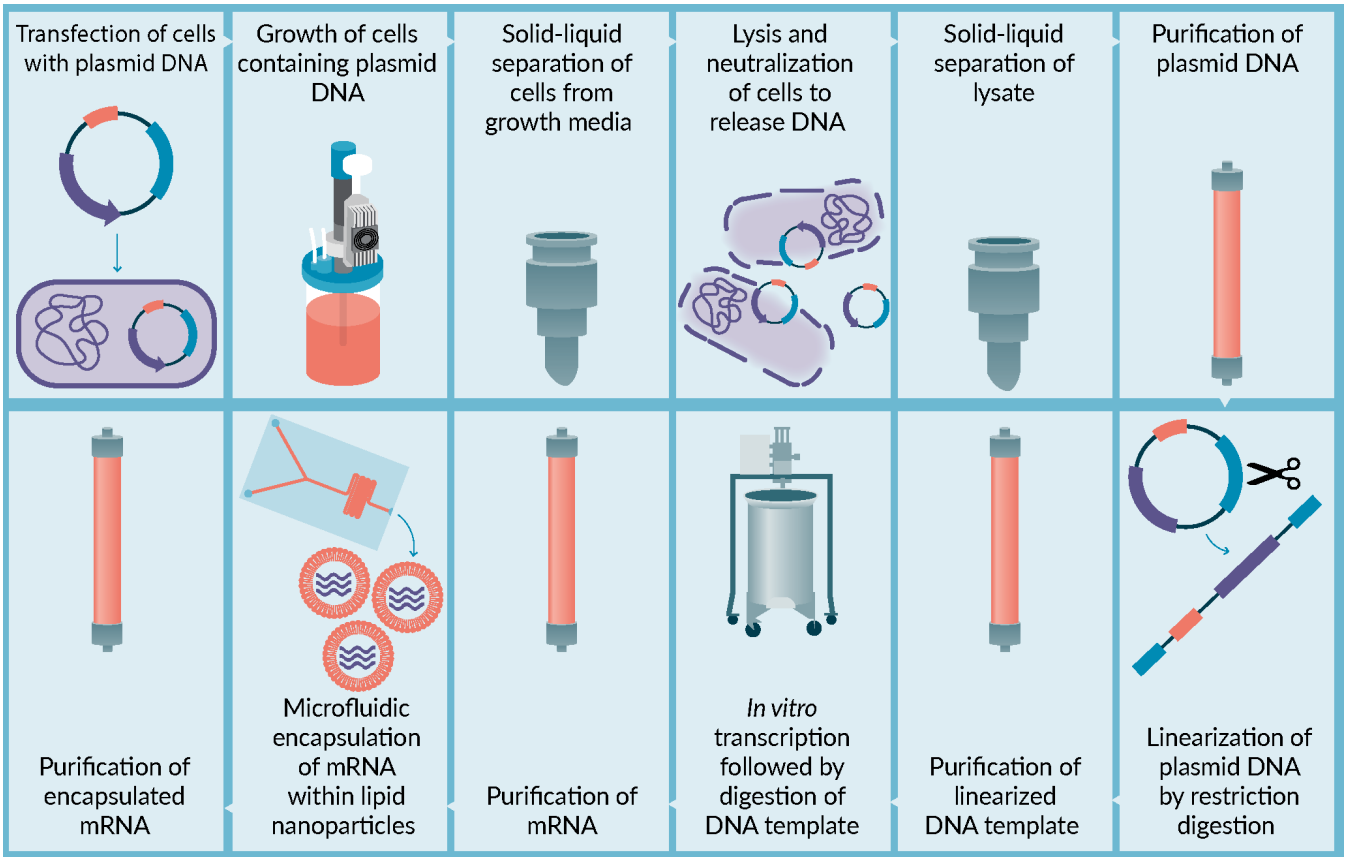
TABLE 1

Comparison of RNA synthesis techniques.

RNA synthesis technique	Advantages	Disadvantages
In vitro transcription	High levels of fidelity and rapid transcription of DNA template [56,57].	T7 polymerase is costly. As a result, some operations may require that the enzyme is manufactured on-site [58].
Oligonucleotide synthesis	The process is cheap and efficient for synthesising short sequences [59].	Only oligonucleotides up to 300 nucleotides long can be synthesised [60].
Cell-based synthesis	The DNA template does not need to be linearized prior to transcription [61].	Transfection of a host organism must occur with the template DNA. Extraction of RNA requires complex procedures [62].

FIGURE 1

A flow diagram of a typical mRNA manufacturing process.



Adapted from [63].

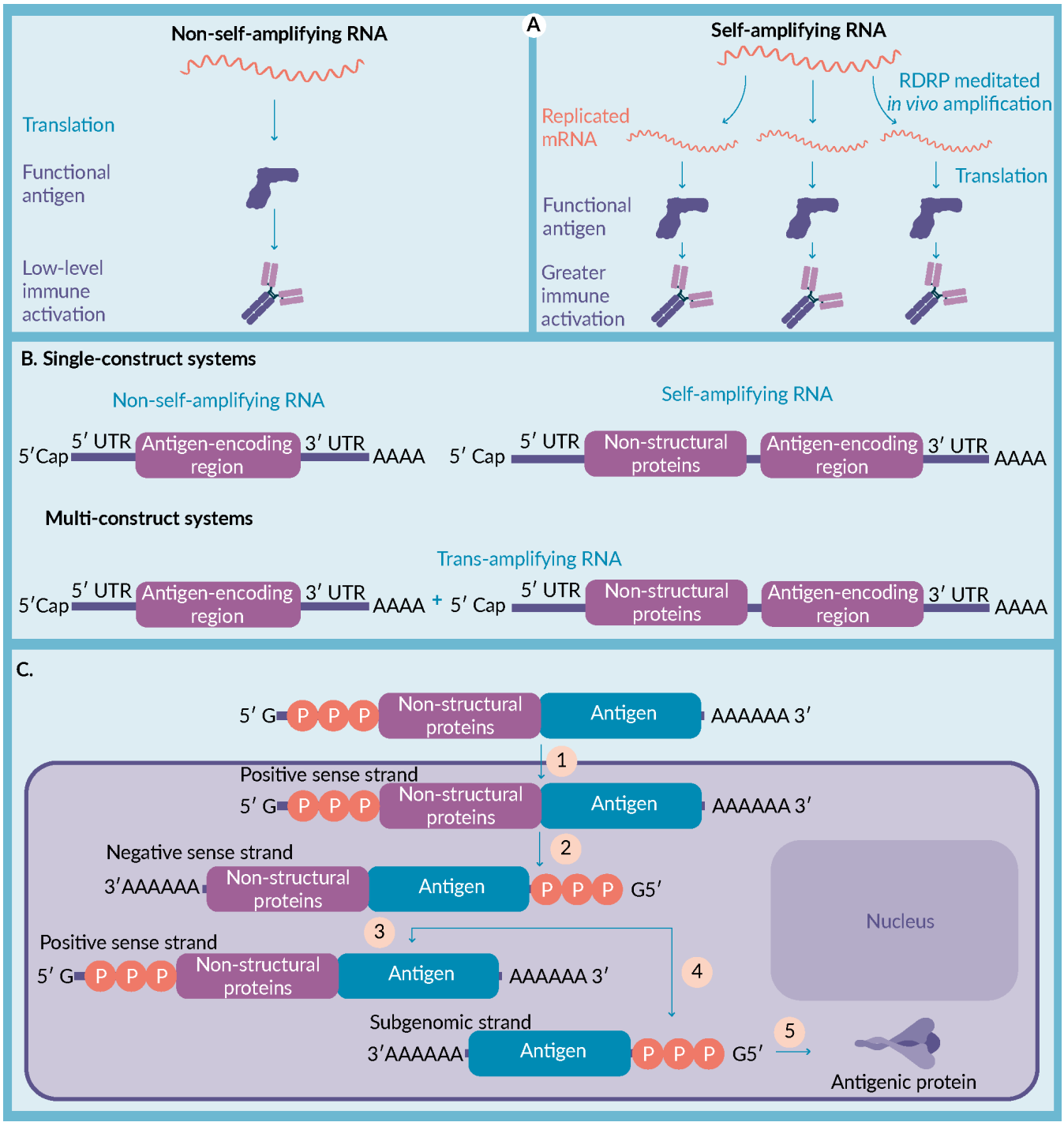
vaccines have been approved by regulatory bodies including the Medicines and Healthcare products Regulatory Agency (MHRA) and the US Food and Drug Administration (FDA) [9].

There are two main types of mRNA, non-replicating mRNA and self-amplifying mRNA (ssmRNA and saRNA, respectively). Both types possess structural similarities, including a 5' cap, 3' and 5' untranslated regions and a polyadenine tail (Poly (A)) [4]. The saRNA contains additional replicons to enable the mRNA to self-replicate, sequences of single-stranded RNA viruses from the genera *Alphavirus, Picornavirus* or *Flavivirus* [10]. These viruses contain a single-stranded, positive-sense genome and can contain regions coding for non-structural proteins (NSPs) (**Figure 2A, C**). Trans-amplifying mRNA (taRNA) is a new type of mRNA vaccine where the mRNA is split into two transcripts, one encoding a peptide of interest and another encoding virally derived replicative machinery. This system is distinct from saRNA, as saRNA contains both the gene of interest and replicase on the same strand. Individual taRNA strands are typically shorter than saRNA and are therefore easier to synthesise. Additionally, taRNA displays greater translational efficiency when compared to saRNA [11,12] **(Figure 2B)**.

The mRNA is in transcribed preferentially in cell-free reactions (*in vitro transcription*, IVT) **(Table 1)** using polymerase enzymes and template DNA [13]. The predominant polymerase is the T7 (T7RNAP) [14], consisting of a single subunit and is highly processive, even in the absence of other transcriptional proteins [15]. T7RNAP exhibits high fidelity, allowing for accurate transcription [68]. In addition to these components, the IVT must also contain nucleotide triphosphates

FIGURE 2

Different types of mRNA.



A: Self-amplifying RNA vaccines induce enhanced immune activation when compared to non-self-amplifying RNA vaccines. Adapted from [64].
B: Structural comparison of single construct systems (saRNA and ssmRNA) against multi-construct systems (taRNA) in the case of potential mRNA vaccinations. Adapted from [12,65].
C: In situ amplification of a self-amplifying RNA construct encoding an antigenic peptide. 1: Transfection of self-amplifying RNA into cell. 2: Transcription of positive-sense strand to create a negative sense strand. 3: Replication of original positive sense strand via transcription of negative sense strand. 4: Transcription of subgenomic region to create subgenomic strand encoding antigen of interest. 5: Translation of subgenomic strand to produce antigen. Adapted from [66].

DOI: 10.18609/cgti.2022.049

(NTPs), polymerase cofactors e.g., $MgCl_2$, polyamine containing buffer and antioxidants [12]. The IVT product yield ($mol_{mRNA}.mol_{pDNA}^{-1}$) and efficiency ($mol_{mRNA}.mol_{impurities/pDNA}^{-1}$), will have an impact on downstream processing steps. The removal of immunogenic product and process related impurities [16] are essential to ensure that mRNA-based prophylactic and therapeutic agents display acceptable levels of efficacy and safety [17].

IMPURITIES PRESENT WITHIN *IN VITRO* TRANSCRIBED mRNA

Process related impurities

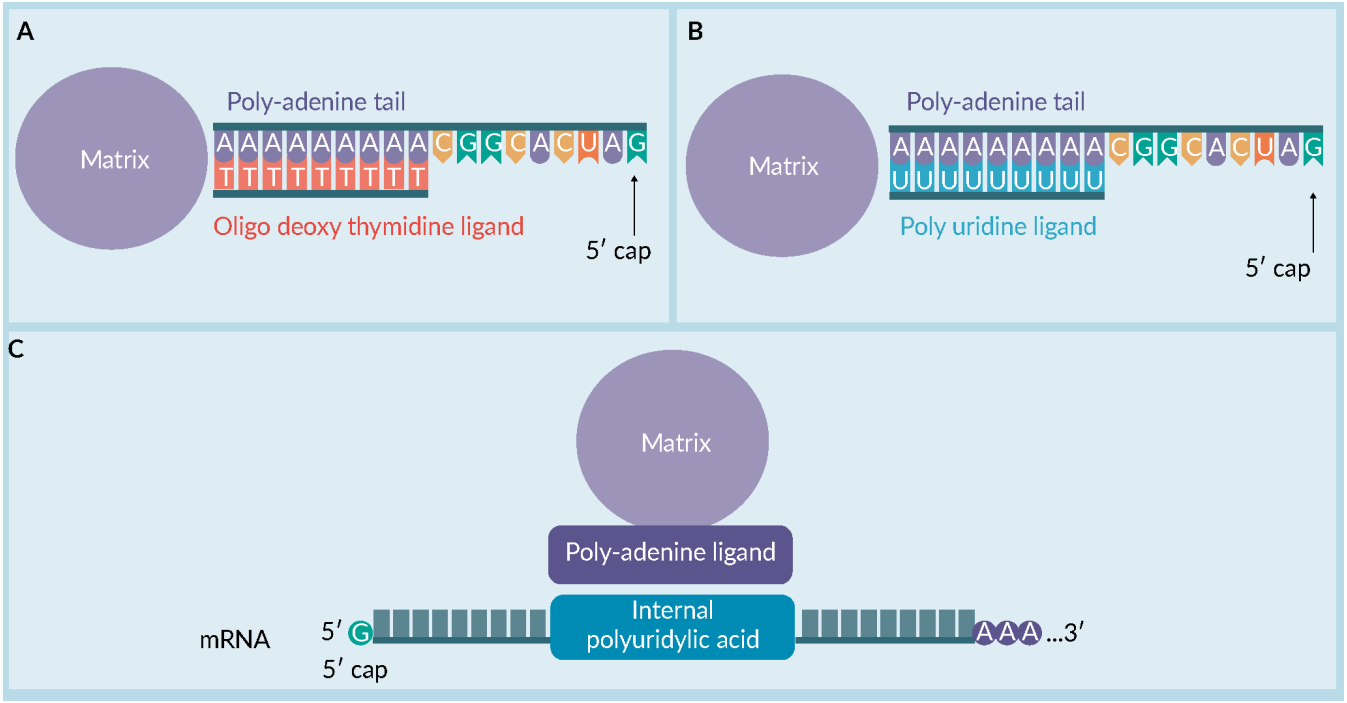
Template DNA

A key concern surrounding template DNA is the potential for genomic integration if plasmids remain intact in the encapsulated mRNA and infiltrate the plasma membrane of cells upon administration [18]. In addition to the threat posed by large fragments, oligonucleotides produced from enzymatic digestion of plasmid DNA (pDNA) may undergo base pairing with partial transcripts to form DNA-RNA hybrid fragments [19].

Plasmids which are produced by microbial fermentation may also contain endotoxins and proteins if they were not removed from the cellular lysate by chromatographic separations prior to IVT. Endotoxin, a lipopolysaccharide constituent of the outer membrane of gram-negative bacteria, has a section that is highly immunogenic (lipid A). Lipid A binds to myeloid differentiation factor 2 and toll-like receptor 4 on the cell surface, initialising signalling pathways, leading to cytokine release and inflammation. As a result of impurities arising from the cell-based synthesis of pDNA, purification steps are necessary prior to IVT [20]. One possible approach to simplify the required purification is the cell-free synthesis of the template DNA [21].

FIGURE 3

Polyadenine tail of mRNA immobilised upon binding.



(A) Polyuridine, (B) oligo deoxythymidine and (C) polyadenine. Adapted from [48,50,67].

RNA polymerase

RNA polymerases, primarily T7, but T3 and SP6 may also be used during IVT will remain in solution unless removed. RNA polymerases are produced through cell-based synthesis and may therefore contain endotoxins. Polymerases may be recognised as foreign antigens upon the binding of complementary antibodies, inducing pro-inflammatory cytokines as part of an adaptive immune response and leading to inflammation [22,23].

Free nucleoside triphosphates

Nucleoside triphosphates that are not incorporated into mRNA during IVT may remain in solution. These free nucleotides may activate neuroinflammatory mechanisms within the central nervous system. The free nucleosides can act as agonists by binding to purinergic receptors (P2), classed into two broad categories: P2X and P2Y. P2X are a group of cation channels which selectively bind adenosine triphosphate while P2Y receptors bind adenosine and uridine triphosphate. A diverse range of P2 receptor types are present in the plasma membranes of macrophages, glial cells, and oligodendrocytes [24].

Product-related impurities

DNA–RNA hybrid fragments

During IVT, RNA synthesis occurs from the 5' to 3' end with synthesis typically beginning at the T7 promoter region. The polymerase, exhibiting high levels of processivity, will continuously transcribe template DNA in cases where the template is not linearised. This continuous transcription may lead to the formation of excessively long transcripts. Linearization by restriction digestion is therefore mandatory to prevent the formation of these long transcripts, providing that suitable restriction sites exist within the construct (Figure 1) [25].

The digestion of linearised DNA creates oligonucleotide fragments, which may undergo base pairing with RNA fragments which are generated as side products of IVT. The association of these fragments with one another leads to the formation of impurities known as DNA-RNA hybrid fragments. The risks of genomic integration, associated with DNA impurities, warrant the removal of hybrid fragments. In addition, the ssRNA component is associated with activation of toll-like receptors 7 and 8, leading to interferon release [18,26]. The fragments may be removed from the IVT mixture using downstream separation techniques, or alternatively can undergo enzymatic digestion when the deoxyribonuclease, DNase1, is added [19].

Partial transcripts

Incomplete RNA transcripts are generated as a by-product during IVT where during transcription initiation, abortive synthesis events occur. As a result, the RNA polymerase produces short mRNA fragments from the template DNA, between 5 and 11 nucleotides long [3]. ssRNA, including partial transcripts, can be detected by toll-like receptors (TLR) 7 and 8. Upon activation, TLR 7 and 8 can induce the release of type 1 interferon.

TABLE 2

Dynamic binding capacities of existing oligo (dT) products.

Product name	Product type	Quoted dynamic binding capacity (mg.mL-1)
Praesto™ Jetted (dT)18-DVB	Beaded chromatography resin	2 (200nt poly (A)) [40]
Poros™ (dT)25	Beaded chromatography resin	0.62 (40nt poly (A)) 4 (2000nt mRNA) 3 (3000nt mRNA) [41]
Dynabeads™ (dT)25	Magnetic beads	0.05 [38]
Sera-Mag™ (dT)14	Magnetic beads	0.11 [39]

DOI: 10.18609/cgti.2022.049

Additionally, nuclear factor kappa B may be activated, as with the activation of RIG1 and MDA5 in the presence of dsRNA [26].

Double-stranded RNA

Double-stranded RNA (dsRNA) may be formed when partial transcripts, formed from abortive transcriptional events, bind to mRNA, and prime the association of transcriptional apparatus with the mRNA. This induces complementary strand synthesis downstream of the site of the transcriptional apparatus binding [16]. A second mechanism of dsRNA synthesis arises from the production of antisense RNA fragments. These fragments are transcribed from the non-coding DNA strand which is found on double-stranded DNA templates. The annealing of antisense fragments to mRNA can occur through the pairing of complementary base sequences. This leads to the generation of dsRNA [16].

dsRNA removal to very low levels from feed material is necessary because the molecule is highly immunogenic [3]. This is illustrated by the molecules ability to induce a cytokine storm in some cases [27,28]. Despite its immunogenicity, dsRNA holds natural biological purposes within human cellular nuclei [29]. However, the entry of dsRNA into the cytosol may induce apoptosis due to its association with viral material [30].

Occurring in all human cells, MDA5 and RIG1 are intracellular receptors. Pathogen associated molecular patterns (PAMPs) can activate MDA5 and RIG1. dsRNA is a PAMP and is often released into the cytosol during viral infection [31]. MDA5 and RIG1 bind differing sizes of dsRNA. Longer strands are bound internally by MDA5, whilst shorter strands are bound at the 5' phosphorylated ends by RIG1 [32,33]. Whilst different sized fragments activate the two receptors, there is overlap in the corresponding signalling pathways. Interferon 1 expression is up-regulated by both MDA5 and RIG1 [31]. A mechanism has also been identified which is dependent on nuclear factor kappa B to stimulate the release of proinflammatory cytokines. Due to the overlapping activities of MDA5 and RIG1, a wide variety of dsRNA strand sizes can be detected through these innate sensing mechanisms [33].

RNase L release within cells is induced by the activation of oligoadenylate synthetase in the presence of dsRNA. Degradation of mRNA may occur in the presence of RNase L. This degradation leads to an inhibition in the translation of mRNA. This mechanism suggests that the removal of dsRNA may contribute to increased levels of mRNA expression. RNase L is also able to cleave dsRNA. The resulting double-stranded fragments may activate intracellular receptors, Melanoma Differentiation Associated Protein 5 (MDA5) and Retinoic Acid Inducible Gene 1 (RIG1) [31].

AFFINITY CHROMATOGRAPHY

Introduction to affinity chromatography

Affinity chromatography was, and continues to be, ubiquitously utilised in the industrial purification of antibodies as a capture step. This is due to its rapid and selective nature [34]. These qualities are also observed in the case of mRNA purification, and it is a highly reliable and consistent primary capture step. The technique supports the use of aqueous buffers and elution may be achieved by simply reducing the salinity of the mobile phase [35]. The technique does not require organic solvents such as acetonitrile in the mobile phase, avoiding the flammability hazard and environmental impact of waste which is produced when compared to reversed phase chromatography [27]. Additionally, mRNA does not require a dedicated tagging step, due to the presence of a poly(A) tail (Figure 2B) [4].

Sodium chloride is used to increase the ionic strength shielding the charge on the ligand and RNA thus allowing the ligand and RNA to bind to each other through base pairing hydrogen bonds. After mRNA is bound to the ligand the salt is removed establishing the original charge repulsion between the

ligand and mRNA. This method allows RNA to unbind from the ligand and be eluted and is known as hybridisation affinity chromatography [27]. The 3' poly(A) tail found on mRNA creates an opportunity for purification by base pair affinity chromatography. Additionally, mRNA can be isolated from transfer and ribosomal RNAs, which do not possess a polyadenine tail [36]. Different affinity ligands can be used in the purification of mRNA, such as oligo deoxythymidine, polyadenine and polyuridine (Figure 3).

Purification of mRNA by affinity chromatography has been shown to enhance the stability of the drug substance. This is due to the high levels of impurity removal that can be achieved. This stability is greater than that of mRNA purified through sedimentation. As a result, affinity chromatography is beneficial to the preservation mRNA as well as patient safety [37].

Oligo deoxythymidine

To effectively tackle the bottlenecks associated with large scale mRNA purification and meet the growing global demand, commercially available products are being developed. Novel products would ideally be compatible with current chromatography platforms, with the current range of oligo deoxythymidine (dT) products aiming to address the low capacity associated with mRNA affinity chromatography (Figure 3A) [12].

Despite rapid development, commercial options for oligo (dT) products are still in their infancy. The main products on the market currently include: Poros (dT)25 and Dynabeads™ (dT)25 by Thermofisher; the CIMmultus™ Oligo (dT)18 monolith and Sera-Mag™ (dT)14 by Cytiva; and Praesto Jetted (dT)18-DVB by Purolite. Both Dynabeads™ (dT)25 and Sera Mag (dT)14 are coated, 1 µm magnetic beads with (dT) ligand coupled onto the surface. The beads boast the high yields and specificity associated with (dT) affinity purification; Dynabeads (dT) has a binding capacity of 10 $\mu g.mg_{resin}^{-1}$, equivalent to 50 $\mu g.mL_{resin}^{-1}$, whereas Sera-Mag (dT) quotes a capacity of 11 $\mu g.mg_{resin}^{-1}$, equivalent to 110 $\mu g.mL_{resin}^{-1}$ [38,39]. The products are ideal for the small-scale purification of polyadenylated mRNA to be used in laboratory techniques, such as RT-PCR and cDNA synthesis. The key benefit of utilising magnetic beads is that the beads can be easily isolated from the supernatant by applying a magnetic field. However, these beads are unsuitable for most large-scale purification platforms as most major purification platforms revolve around a form of fixed bed column chromatography where a packed bed, monolith or membrane would be used. The 1µm bead size would cause large backpressure if used in a packed bed and is unlikely to be considered in industrial applications, except for fluidised bed systems. Table 2 summaries the capacities of existing oligo (dT) products quoted by manufacturers.

The main options for industrially appropriate oligo (dT) products are the Praesto Jetted (dT)18-DVB, Poros (dT)25 and CIMmultus (dT)18 monolith. The first two are resin technologies that utilise a divinyl benzene base matrix and affix the (dT) ligand to the surface with a proprietary linker. Purolite have released a binding capacity of 2 $mg.mL_{resin}^{-1}$ of 200 nt Poly(A) compared to 0.62 $mg.mL_{resin}^{-1}$ of 40 nt Poly(A) on a Poros (dT)25 certificate of analysis [40,41] The Poros (dT) displays 10% breakthrough values of 4 and 3 $mg.mL_{resin}^{-1}$ capacity for 2,000 and 3,000 nt mRNAs, respectively, whilst 1,000 nt mRNAs show a 5% breakthrough of 4 $mg.mL_{resin}^{-1}$ [42]. A clear correlation between mRNA size and capacity is observed, with Poros (dT)25 having lower capacity for larger mRNAs. This indicates that surface crowding is preventing the full utilisation of the bound (dT) ligand. Despite the reduced capacity for larger mRNAs, the resin can be reused for 10 cycles with only a marginal drop in yield.

Commercially available monoliths include the CIMmultus (dT)18 range from BIA Separations. These are Poly glycidyl methacrylate-co-ethylene dimethacrylate monoliths where (dT)18 is immobilised with a C6 or C12 linker chain. The product exhibits a

DOI: 10.18609/cgti.2022.049

ligand density of 0.5 $mg_{Oligo\ (dT)}.mL_{wet\ support}^{-1}$. There is currently no available data for capacity with any length of mRNA [43,44]. However, a 1 mL CIMmultus™ Oligo (dT) is capable of an 80% recovery when purifying an IVT mixture containing approximately 180µg 2000 nt mRNA. Additionally, monolith separations can be completed in a shorter space of time due to the higher rates of convective flow [45].

Comparing existing products will remain challenging until capacity data for a wide range of mRNA constructs is released. The (dT)18 ligand present on the Praesto Jetted (dT)18-DVB indicates a capacity somewhat like Poros (dT)25. The comparison becomes difficult when accounting for differences in the resin and monolith technology. Each technology presents options for mRNA purification at an industrial scale. A second generation of products is required to further push the boundaries in capacity. New options could include other base materials, such as agarose. Agarose (dT)20 was prepared using NHS activated Sepharose FF. This achieved a 1.6 $mg.mL_{resin}^{-1}$ capacity with a 900 nt polyadenylated mRNA [46]. However, this could be indicative of the unsuitability of agarose as a base matrix at relatively large pore sizes, given that no agarose products are yet commercially available.

Polyuridine

A capture modality somewhat akin to oligo (dT), polyuridine (poly(U)) targets the poly(A) tail of mRNA for capture via hybridisation (Figure 3B). The ligand consists of a chain of uridine nucleotides and may be immobilised on a medium such as Sepharose. Phillips et al [47] demonstrated high levels of binding specificity between poly(U) and poly(A) containing mRNA. Over 90% of binding in poly(U) agarose columns was complementary. Non-complementary binding was 3% or lower. Only poly(A) tails effectively initiated binding, with internal and non-sequential adenylated sequences not binding to the columns. The technique may be particularly valuable in isolating mRNA with a short poly(A) tail, as only 10 nucleotides in the tail were sufficient for binding to occur to a detectable extent. This suggested a high binding affinity between the target and ligand. This binding frequency increased up to 25 nucleotides, after which binding was independent of poly(A) length.

The high binding affinity of poly(U) presents itself as an advantageous characteristic for the purification of mRNA. However, this property may prevent its implementation in many cases. Berman, Gornaeva and Mazurov [48] showed that an irreversible and non-specific binding of RNA occurred when poly(U) was used on a Sepharose matrix. Strong adsorption of the target may require the use of extreme elution conditions and the addition of compounds which counteract the effects of non-specific binding. Ochoa, Kempf and Egly [49] demonstrated that poly(A) RNA does not exhibit significant binding affinity for Sepharose in the absence of poly(U) when comparable conditions are provided. This suggests that non-specific binding may arise from the ligand itself, or from structures related to the functionality of said ligand. SDS, an anionic surfactant, was shown to be highly effective at eluting poly(A) mRNA from poly(U). This suggested that hydrophobic interactions could be attributed in part to non-specific binding between the target and immobilised ligand.

Chaotropic salts may be an ineffective constituent of elution buffers in the case of poly(U), as their use does not guarantee effective unbinding. Additionally, the potential for the formation of secondary structures in their presence exists [49]. It is unclear why disparities exist in the frequency of non-specific binding events between studies. The exact contributions of binding mechanisms to the unfavourable elution requirements of poly(U) have not yet been fully ascertained. A potential future approach to irreversible adsorption of the target may involve reducing the overall ligand length so that nonspecific binding events are reduced.

Polyadenine

An alternative and seldom utilised mode of affinity separation applies a polyadenine ligand to capture RNA fragments containing internal polyuridine sequences (Figure 3C). This ligand is distinct from both oligo (dT) and poly(U), as separation does not rely on interactions with the poly(A) tail found on mammalian mRNA [50].

Poly(A) is only effective at isolating mRNAs with oligo U sequences. This suggests that some targets may not be suitable for capture by this step as they may lack the necessary poly(U) sequences. Poly(U) sequences are not ubiquitous in naturally occurring cytoplasmic mRNA and approximately 20% of poly(A) containing mRNA also contains internal oligo(U) sequences. The poly(A) tail found on mRNA presents a more broadly applicable purification opportunity than internal poly(U) sequences if oligo(U) sequences are not deliberately inserted when producing synthetic mRNA [51]. Polyadenine may emerge as an effective method for the isolation of mRNA targets containing internal poly(U) sequences. However, it is not currently utilised at industrial scales for the purposes of therapeutic or prophylactic mRNA purification.

Elution conditions

Extremes in pH or chaotropic agents are capable of disrupting hydrogen bonding and causing elution [52]. Binding affinity between the target and immobilised ligand is affected by both the pH and salinity of the buffer solution. Highly acidic or alkaline conditions induce disruption of hydrogen bonds, therefore reducing the binding affinity between complementary bases. However, mRNA may incur damage under extreme pH values. Cleavage of phosphodiester bonds in RNA is probable at pH > 6 (alkaline hydrolysis) and pH < 2 (acid hydrolysis) [53]. Existing affinity products typically elute mRNA at a close to neutral pH, instead relying on a lowered salt concentration to induce unbinding [38–45].

Binding affinity between bases increases with salinity. Association of positive ions with phosphate groups present on the mRNA has a stabilising effect. This is because the repulsion between the negative phosphate groups is reduced [35].

LIMITATIONS OF AFFINITY CHROMATOGRAPHY

Affinity chromatography as provided by ligands like oligo (dT) provides a method of selectively binding mRNA molecules which contain a 3' poly(A) tail. The poly(A) tail is required to reduce mRNA *in vivo* degradation rates to make effective therapies. This separation method allows mRNA with a poly(A) tail to be isolated from IVT related impurities, including excess nucleosides, residual enzymes, excess capping reagents and buffer components. It will also only separate mRNA molecules with a poly(A) tail so that incomplete transcripts lacking the poly(A) tail, required for *in vivo* stability, are not bound by the media. Oligo (dT) affinity chromatography will not provide a method to separate mRNA species lacking a 5' cap (required to avoid innate immune system activation). Also, it will not separate double-stranded RNA formed through reactions discussed earlier. Double-stranded mRNA is an important critical quality attribute for mRNA, therefore, further polishing separations are required to meet specifications necessary for dsRNA removal.

As well as these quality attribute limitations for oligo (dT) chromatography, there are chromatographic limitations. mRNA molecules as discussed above are large (approximately 4000nt) and in the case of saRNA very large (>10,000nt). These are species with molecular weights of approximately 2MDa–5MDa with the same dimensional range as virus particles. As such, the poly(A) tail utilized in binding an affinity ligand is a small component of a large particle. Hence binding kinetics have the potential to be slow due to steric factors. Binding capacity may be limited, requiring a large volume of affinity media to purify a given amount of

DOI: 10.18609/cgti.2022.049

mRNA. This will apply particularly to beaded media where mRNA is likely to only bind to surface ligands and very little adsorption occurs to ligands contained within pores. Alternative stationary phase design – membranes, monoliths or nanofibers are likely to achieve higher binding capacities by increasing mRNA access to ligands. A further limitation arises from the need to increase solution ionic strength to achieve binding. Salt precipitation is itself a viable method of separating mRNA and hence there is a fine balance between precipitating mRNA and promoting oligo (dT) ligand binding [3].

Alternatives to affinity chromatography need to consider the two factors discussed above – achieving critical quality attributes in terms of control of product related impurities and the potential for low capacities in bind and elute chromatography. Achieving both these objectives with a single approach is currently difficult. Control of product related impurities such as dsRNA has been described using reversed phase high performance chromatography (RP-HPLC) [54]. While this has been shown to be effective in control of dsRNA levels, the approach uses beaded media which will have capacities limited by accessible surface area and requires the use of acetonitrile in the mobile phase.

Effective removal of process related impurities can be achieved by flow-through chromatography. The use of media combining a surface layer, preventing large molecules like mRNA entering the pores of a core containing mixed mode or hydrophobic media has been described using media such as Cytiva CaptoCore chromatography resin [55]. As a flow through media there are no limitations presented by the binding of the mRNA, the limitations are provided by the impurity species. While removal of transcriptional impurities can be achieved, such an approach is unlikely to control levels of dsRNA, which will also flow through.

The inability of affinity chromatography to effectively separate product-related impurities from the target creates the requirement for polishing steps. With each additional unit operation, the overall yield of mRNA is reduced, and process efficiency decreases. It is therefore economically beneficial to utilise separation techniques which distinguish between product-related impurities with high resolution. Yield varies between mRNA constructs and buffer composition, however, CIMmultus (dT)18 is quoted at approximately 80% and Praesto Jetted (dT) 18-DVB at approximately 60% [43,70]. Over-reliance on the poly(A) tail as a basis of separation ignores the potential formation of secondary structures, DNA-RNA hybrid fragments and partial transcripts which may display internal or external poly(A) sequences [3].

CONCLUSIONS & FUTURE PERSPECTIVES

mRNA is a therapeutic and prophylactic modality with the potential for rapid development, cell-free manufacture, and stability once highly purified. Impurities generated through *in vitro* transcription, the dominant synthesis technique, are categorised as process and product related. The removal of both sets of impurities is essential to the safety, functionality, and stability of mRNA in a clinical setting. Critical quality attributes such dsRNA must be removed to suitably low levels to comply with the relevant regulatory guidance. When developing new products, the market authorisation holder may be required to carry out assays such as immunoblotting to prove that impurity species are below detectable levels. Immunoblotting was required by the European Medicines Agency to verify the removal of dsRNA from the BNT162b2 (COMIRNATY®) COVID-19 vaccine [69].

Affinity chromatography is an attractive primary capture step for the removal of product and process related impurities, but current approaches struggle to separate the target from structurally similar product related impurities. The benefits of affinity chromatography include purification at ambient temperatures, the use of aqueous buffers in the mobile phase and elution with a simple salt gradient. mRNA affinity

chromatography in industry relies almost exclusively on oligo deoxythymidine ligand chemistries, however, polyuridine and polyadenine ligands have both been implemented at the bench scale.

Oligo deoxythymidine products include beaded resins, monoliths, membranes, and magnetic particles. Polyuridine may be susceptible to strong non-specific adsorption due to hydrophobic interactions, although this may be addressed through alterations in buffer composition. Polyadenine as an immobilised ligand is only applicable to targets with internal uridine sequences and does not rely on the 3' poly adenine tail of mRNA, unlike other modes.

These mRNA affinity-based methods have not yet become the default capture step protein A affinity chromatography is for mAb products. The relatively simple range of process impurities from upstream IVT and the large size of mRNA indicates that options such tangential flow filtration may compete. Equally there is potential to develop novel affinity ligands to improve capacity, and ideally selectivity for product related impurities. It is also likely there is value in pursuing novel matrix materials that allow greater accessibility to ligands for relatively large products such as mRNA.

Further, as novel mRNA targets and modalities emerge, affinity chromatography will need to adapt to the array of strand lengths and associated impurities which will be encountered during purification. Capacities are typically found to be greater for smaller constructs, interactions between the target and ligand must be characterised and mRNA secondary structures identified to improve media for larger constructs. Perhaps most importantly effective purification also requires removal of product-related impurities, particularly dsRNA due to their immunostimulatory effects.

REFERENCES

1. Jackson N, Kester K, Casimiro D, Gurunathan S, DeRosa F. The promise of mRNA vaccines: a biotech and industrial perspective. npj *Vaccines*. 2020; 5
2. Bown C, Bollyky T. How COVID-19 vaccine supply chains emerged in the midst of a pandemic. *World Econ*. 2021; 2021; 45, 468.
3. Baiersdörfer M, Boros G, Muramatsu H et al. A Facile Method for the Removal of dsRNA Contaminant from in vitro-Transcribed mRNA. *Mol. Ther. Nucleic Acids* 2019; 2020;15, 26–35.
4. Kowalski P, Rudra A, Miao L, Anderson D. Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery. *Mol. Ther.* 2019; 27, 710–728.
5. Wolff J, Malone R, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. *Science* 1990; 247, 1465–1468.
6. Suschak J, Williams J, Schmaljohn C. Advancements in DNA vaccine vectors, non-mechanical delivery methods, and molecular adjuvants to increase immunogenicity. *Hum. Vaccin. Immunother.* 2017; 13, 2837–2848.
7. Jäschke A, Helm M. RNA Sex. Chem. Biol. 2003; 10, 1148–1150.
8. Yamamoto A, Kormann M, Rosenecker J, Rudolph C. Current prospects for mRNA gene delivery. Eur. J. Pharm. Biopharm. 2009; 71, 484–489.
9. Moore J, Millar B. Improving COVID-19 vaccine-related health literacy and vaccine uptake in patients: Comparison on the readability of patient information leaflets of approved COVID-19 vaccines. J. Clin. Pharm. Ther. 2021;46, 1498–1500.
10. Wadhwa A, Aljabbari A, Lokras A, Foged C, Thakur A. Opportunities and Challenges in the Delivery of mRNA-Based *Vaccines*. Pharmaceutics 2020; 12(2): 102.
11. Beissert T, Perkovic M, Vogel A et al. A Trans-amplifying RNA Vaccine Strategy for Induction of Potent Protective Immunity. *Mol. Ther.*2020; 28(1): 119.
12. Rosa S, Prazeres D, Azevedo A, Marques M. mRNA vaccines manufacturing: Challenges and bottlenecks. *Vaccine* 2021; 39(16): 2192.
13. Beckert B, Masquida B. Synthesis of RNA by in vitro Transcription. *RNA* 2010; 703: 29.
14. Conrad T, Plumbom I, Alcobendas M, Vidal R, Sauer S. Maximizing transcription of nucleic acids with efficient T7 promoters. *Commun. Biol.* 2020; 3(1): 1.
15. Borkotoky S, Murali A. The highly efficient T7 RNA polymerase: A wonder

DOI: 10.18609/cgti.2022.049

macromolecule in biological realm. *Int. J. Biol. Macromol* 2018; 118(1): 49.

16. Wu M, Asahara H, Tzertzinis G, Roy B. Synthesis of low immunogenicity RNA with high-temperature in vitro transcription. RNA 2020; 26(3): 345–360.

17. Weissman D, Pardi N, Muramatsu H, Karikó K. HPLC Purification of in vitro Transcribed Long RNA. *Methods Mol. Biol* 2013; 969: 43.

18. Xia J, Lu G, Lu J, Zhang J, Feng L, Wang B. Towards an effective mRNA vaccine against 2019-nCoV: demonstration of virus-like particles expressed from a modified mRNA cocktail. *ChinaXiv* 2020; : 2.

19. Stöcher M, Berg J. Removal of template DNA from cRNA preparations by combined oligo(dT) affinity chromatography and DNase I digestion. *BioTechniques* 2004; 36(3): 480.

20. Bohannon J, Hernandez A, Enkhbaatar P, Adams W, Sherwood E. The Immunobiology of Toll-Like Receptor 4 Agonists. *Shock* 2013; 40(6): 451.

21. Adie T, Orefo I, Kysh D et al. dbDNA™: An advanced platform for genetic medicines. Drug Discovery Today 2022; 27(2): 374.

22. Chaplin D. Overview of the immune response. Journal of Allergy and Clinical Immunology 2010; 125(2): 3–23.

23. Green M, Sambrook J. Synthesis of Single-Stranded RNA Probes by in vitro Transcription. *Cold Spring Harb. Protoc.* 2020; 2020(1).

24. Weisman G, Camden J, Peterson T, Ajit D, Woods L, Erb L. P2 Receptors for Extracellular Nucleotides in the Central Nervous System: Role of P2X7 and P2Y2 Receptor Interactions in Neuroinflammation. *Mol. Neurobiol.* 2012; 46(1): 96–113.

25. Ohanube G, Obeta U. Generation of mRNA Vaccine: An Analysis of Two Types of Vectors. International Journal Of Pharmaceutical And Phytopharmacological Research 2021; 11(4): 25.

26. Bender A, Tzvetkov E, Pereira A et al. TLR7 and TLR8 Differentially Activate the IRF and NF-κB Pathways in Specific Cell Types to Promote Inflammation. *ImmunoHorizons* 2020; 4(2): 93.

27. Gagnon P, Goričar B, Peršič Š, Černigoj U, Štrancar A. Two new capture options for improved purification of large mRNA. *Cell and Gene Therapy Insights* 2020; 6(7): 1035.

28. Kasumba D, Grandvaux N. Therapeutic Targeting of RIG-I and MDA5 Might Not Lead to the Same Rome. Trends in Pharmacological *Science*s 2019; 40(2): 117.

29. Portal M, Pavet V, Erb C, Gronemeyer H. Human cells contain natural double-stranded RNAs with potential regulatory functions. *Nat. Struct. Mol. Biol.* 2014; 22(1): 89.

30. Gantier M, Williams B. The response of mammalian cells to double-stranded RNA. *Cytokine & Growth Factor Rev.* 2007; 18(6): 363.

31. Brisse M, Ly H. Comparative Structure and Function Analysis of the RIG-I-Like Receptors: RIG-I and MDA5. *Front. Immunol.* 2019; 10(1): 3.

32. Reikine S, Nguyen J, Modis Y. Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5. *Front. Immunol.* 2014; 5: 342.

33. Saito T, Gale M. Differential recognition of double-stranded RNA by RIG-I–like receptors in antiviral immunity. *J. Exp. Med.* 2008; 205(7): 1524.

34. Arora S, Saxena V, Ayyar B. Affinity chromatography: A versatile technique for antibody purification. Methods 2017; 116: 84.

35. Farrell R. Isolation of Polyadenylated RNA. RNA Methodologies 2017; 5: 527–544.

36. Slater R. The Purification of Poly(A)-Containing RNA by Affinity Chromatography. Nucleic Acids; 2: 117.

37. Korenč M, Mencin N, Puc J et al. Chromatographic purification with CIMmultus™ Oligo dT increases mRNA stability. Cell and Gene Therapy Insights 2021; 7(9): 1207.

38. ThermoFisher Scientific. Dynabeads™ Oligo (dT)25 [Internet]. 1st ed. ThermoFisher Scentific; 2020. p. 1–2.

39. 39. Cytiva. Sera-Mag Oligo(dT)-Coated Magnetic Particles [Internet]. 1st ed. Cytiva; 2020. p. 1–2.

40. Purolite. Praesto Jetted (dT)18- DVB [Internet]. 1st ed. Purolite Ltd; 2022. p. 1–2.

41. ThermoFisher Scientific. POROS™ Oligo (dT)25 Affinity Resin [Internet]. 1st ed. ThermoFisher Scientific; 2020. p. 1–8.

42. Flook K. Supporting development of mRNA-based therapies by addressing large-scale purification challenges. Cell and Gene Therapy Insights 2021; 7(5): 489–502.

43. BIA Separations. CIMmultus™ Oligo dT18 1 mL Monolithic Column (C6 Linker) (2 µm channels) [Internet]. 1st ed. BIA Separations; 2020. p. 1.

44. BIA Separations. BIA Separations, 2020. CIMmultus™ Oligo dT18 1 mL Monolithic Column (C12 Linker) (2 µm channels). 1st ed. BIA Separations; 2020. p. 1.

45. BIA Separations. Purification of messenger RNA by affinity chromatography on CIMmultus™ Oligo (dT) column

[Internet]. 1st ed. BIA Separations; 2020; 2.

46. Issa, W. J.; Aunins, J. G.; Bancel, S. Ribonucleic Acid Purification. WO2014152031A1, September 25, 2014.

47. Phillips L, Pang R, Park J, Hollis V, Famuyiwa F. Poly(U)-Agarose Affinity Chromatography: Specific, Sensitivity Selectivity, and Affinity of Binding. Preparative Biochemistry 1980; 10(1): 11.

48. Berman A, Gornaeva N, Mazurov V. Biospecific chromatography of poly(A)-containing RNA on poly(U)-Sepharose. Biokhimiia 1978; 43(10): 1830.

49. Ochoa J, Kempf J, Egly J. Interaction of poly(A) with different adsorbents for affinity chromatography of nucleic acids. *Int. J. Biol. Macromol* 1980; 2(1): 33–38.

50. Wood W, Edmonds M. A method for isolation of oligo(uridylic acid)-containing messenger ribonucleic acid from Hela cells. Biochemistry 1981; 20(19): 5359.

51. Korwek E, Nakazato H, Venkatesan S, Edmonds M. Poly(uridylic acid) sequences in messenger ribonucleic acid of HeLa cells. *Biochemistry* 1976; 15(21): 4643.

52. Farrah S, Shah D, Ingram L. Effects of chaotropic and antichaotropic agents on elution of poliovirus adsorbed on membrane filters. *PNAS* 1981; 78(2): 1229–1232.

53. Bernhardt H, Tate W. Primordial soup or vinaigrette: did the RNA world evolve at acidic pH?. *Biol. Direct.* 2012; 7(1): 4.

54. Karikó K, Muramatsu H, Ludwig J, Weissman D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res.* 2011; 39(21): 142.

55. Berlanda S, Wen Y, Geall A, Porter F. RNA Purification Methods. WO 2014/140211, 2018.

56. Reid-Bayliss K, Loeb L. Accurate RNA consensus sequencing for high-fidelity detection of transcriptional mutagenesis-induced epimutations. Proceedings of the National Academy of *Sciences* 2017; 114(35): 9415–9420.

57. Ali M. Efforts to Develop Rapid Technology of in vitro Transcription and Translation in Vaccine Synthesis in Indonesia. Indonesian Bulletin of Animal and Veterinary *Sciences* 2016; 25(4): 1.

58. Gallo S, Furler M, Sigel R. in vitro Transcription and Purification of RNAs of Different Size. CHIMIA *Int. J. Chem.* 2005; 59(11): 813.

59. 59.de Koning M, Ghisaidoobe A, Duynstee H, Ten Kortenaar P, Filippov D, van der Marel G. Simple and Efficient Solution-Phase Synthesis of Oligonucleotides Using Extractive Work-Up. *Org. Process Res. Dev.* 2006; 10(6): 1238.

60. Song L, Deng Z, Gong Z, Li L, Li B. Large-Scale de novo Oligonucleotide Synthesis for Whole-Genome Synthesis and Data Storage: Challenges and Opportunities. *Front. Bioeng.* 2021; 9: 2.

61. Štrancar A. High yield mRNA production process from E.Coli to highly pure mRNA. Presentation presented at; 2020; *Bio Process International.*

62. Tinafar A, Jaenes K, Pardee K. Synthetic Biology Goes Cell-Free. *BMC Biol.* 2019; 17(1): 1,8.

63. Ouranidis A, Vavilis T, Mandala E et al. mRNA Therapeutic Modalities Design, Formulation and Manufacturing under Pharma 4.0 *Principles. Biomedicines* 2021; 10(1): 50.

64. Vogel A, Lambert L, Kinnear E et al. Self-Amplifying RNA *Vaccines* Give Equivalent Protection against Influenza to mRNA *Vaccines* but at Much Lower Doses. *Mol. Ther.*2018; 26(2): 446.

65. Rodriguez-Gascon A, del Pozo-Rodríguez A, Solinís M. Development of nucleic acid vaccines: use of self-amplifying RNA in lipid nanoparticles. *Int. J. Nanomedicine* 2014; 10(9): 1836.

66. Bloom K, van den Berg F, Arbuthnot P. Self-amplifying RNA vaccines for infectious diseases. Gene Therapy 2020; 28: 117.

67. Sau S, Larsen A, Chaput J. Automated solid-phase synthesis of high capacity oligo-dT cellulose for affinity purification of poly-A tagged biomolecules. *Bioorg. Med. Chem.* 2014; 24(24): 5692–5694.

68. Ulrich S, Kool E. Importance of Steric Effects on the Efficiency and Fidelity of Transcription by T7 RNA *Polymerase. Biochemistry* 2011; 50(47): 10343.

69. 2021. Assessment report Comirnaty. 1st ed. [ebook] EMEA, p.40.

70. 2021. Praesto® Jetted (dT)18-DVB. 1st ed. [ebook] Purolite, pp.4, 6.

AFFILIATIONS

Julian Grinsted,
PhD student
University College London
United Kingdom

John Liddell,
Chief Technologist
Centre for Process Innovation

Emir Bouleghlimat,
Research Scientist,
Purolite Corporation

DOI: 10.18609/cgti.2022.049

Ka Yan Kwok,
Alumna of University College London
United Kingdom

Georgia Taylor,
PhD student
University College London
United Kingdom

Marco P C Marques
Lecturer in Biochemical Engeneering
University College London
United Kingdom

Daniel G Bracewell,
Professor of Bioprocess Analysis,
University College London
United Kingdom

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Grinsted J is a PhD Student at University College London, supported by EPSRC and Purolite industrial CASE studentship (grant: EP/T517793/1). Liddell J is an employee of the Centre for Process Innovation. Bouleghlimat E is an employee of Purolite. Kwok KY is an alumna of University College London. Taylor G is a PhD Student at University College London (grant: EP/T517793/1). Marques PCM is a Lecturer in Biochemical Engineering at UCL, supported by the Future Biomanufacturing Research Hub (grant: EP/S01778X/1). Bracewell DG is a Professor of Bioprocess Analysis, University College London. The authors have no other conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Feb 09 2022;* ***Revised manuscript received:*** *Mar 09 2022;* ***First published:*** *Mar 23 2022;* ***Corrected article published:*** *Jan 09 2023*