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Adventitious agent detection methods in bio-pharmaceutical applications with a focus on viruses, bacteria, and mycoplasma

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Adventitious agents present significant complications to biopharmaceutical manufacturing. Adventitious agents include numerous lifeforms such as bacteria, fungi, viruses, mycoplasma, and others that are inadvertently introduced into biological systems. They present significant problems to the stability of cell cultures and the sterility of manufacturing products. In this review, detection methods for bacteria, viruses, and mycoplasma are comprehensively addressed. Detection methods for viruses include traditional culture-based methods, electron microscopy studies, *in vitro* molecular and antibody assays, sequencing methods (massive parallel or next generation sequencing), and degenerate PCR (polymerase chain reaction). Bacteria, on the other hand, can be detected with culture-based approaches, PCR, and biosensor-based methods. Mycoplasma can be detected via PCR (including specific kits), microbiological culture methods, and enzyme-linked immunosorbent assays (ELISA). This review highlights the advantages and weaknesses of current detection methods while exploring potential avenues for further development and improvement of novel detection methods. Additionally, a brief evaluation of the transition of these methods into the gene therapy production realm with a focus on viral titer monitoring will be presented.

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Introduction

The World Health Organization (WHO) defines adventitious agents as microorganisms that have been unintentionally introduced into the manufacturing process of a biological medicine [1]. These microorganisms can be introduced throughout the manufacturing process including through starting materials and via human intervention [1]. Thus, regulatory agencies require testing for adventitious agents at various stages of the process. If an adventitious agent is detected, it is important to determine its species, origin, and evaluate its potential for human infection [1]. However, despite extensive efforts to detect adventitious agents, some have still been found in pharmaceutical products such as the presence of Porcine Circovirus 1 (PCV1) in a commercial rotavirus vaccine and novel rhabdovirus in the Sf9 cell line [2–4]. Thus, it is still challenging to efficiently detect adventitious agents using conventional methods.

It is important to note that none of the currently available methods represents a true, 'ideal' detection method. The 'ideal' detection method would be able to use minimal sample, detect all known adventitious agents and be able to identify potentially unknown agents, have a small limit of detection, be inexpensive, and be able to produce results in real-time or very rapidly. The required limit of detection will depend upon the agent. For example, well known human pathogens must be detected at smaller LODs to ensure patient safety and to meet regulatory requirements [1]. Thus, the development of novel, sensitive detection methods is of paramount importance to the pharmaceutical industry. The aim of this review is to assess traditional and novel detection methods for adventitious agents. These detection methods will range from simplistic to highly developed techniques. In addition, the adaptation of these methods from adventitious agent detection to use in other applications such as gene therapeutics production will be briefly noted.

Traditional detection methods

Traditionally, bacteria and mycoplasma have been detected by culturing a sample of supernatant on agar medium [6]. Bacteria and mycoplasma may also be detected following the inoculation of embryonic chicken eggs via the yolk sac route [7]. Viruses, however, have been traditionally detected via animal inoculation [5]. Additionally, cell culture may also reveal virus contamination if cytopathic effects are observed. Finally, a hemadsorption (HAD) test may be performed, but this test can only be performed on specific virus types [8,9]. These traditional detection methods for viruses,

mycoplasma, and bacteria are slow, laborious processes. Furthermore, these methods often do not identify the contaminating agent but simply confirm that an agent is present. Also, without the use of an established laboratory, labor and equipment costs to perform cell culture or animal inoculation tests can become expensive.

Microscopy detection methods

Microscopy techniques are often used in parallel to traditional methods as a complementary or orthogonal detection method. Historically, bacteria have been directly observed using light microscopes whereas the effect of viruses on cell phenology has been observed as an indirect detection method [11,12••]. Finally, mycoplasma cannot be seen when the optics of a light microscope are focused on the cell monolayer but may be seen at the air/medium interface [12••]. However, in recent years, more advanced microscopy detection methods have been developed for the detection of bacteria, mycoplasma, and viruses.

Electron microscopy

Electron microscopy has existed since the 1930s and has been used for the study of viruses since that time [13•]. Transmission electron microscopy (TEM) offers higher resolution than traditional light microscopy and is the only imaging technique that allows for the direct visualization of viruses [14••]. TEM can be used to document the presence of retroviruses and retrovirus-like particles and gauge the concentration of viral particles [14••]. Thus, regulatory agencies have required the use of orthogonal methods to confirm the presence of contaminants including the use of TEM [12••,14••]. Additionally, TEM has proven essential in identifying novel viruses and sub-types of viruses [12••].

Images from TEM enable for exhaustive analysis for virus particles and present a 'catch-all' method for identifying adventitious viruses [13•]. However, this analysis is labor intensive. Thus, a novel method presented by Ito *et al.* uses a fully convolutional neural network (FCN) approach to detect viral particles from TEM images [13•]. Based on Ito *et al.*, this FCN detection method outperformed similar methods used for the detection of viral particles within TEM images. As a complementary technique to TEM, immuno-electron microscopy (IEM) can be used for virus identification [15]. IEM can work directly with raw serum which minimizes sample preparation time. Thus, electron microscopy provides a method for detection and identification of adventitious virus particles.

However, microscopy techniques tend to have higher limits of detection and are highly dependent on the homogeneity of the medium being sampled. Additionally, exceedingly small samples are used for detection, and, therefore, only a small fraction (or none) of a specific contaminant may be in the field of view if the concentration of the contaminant is exceptionally low. If, however,

Table 1

Adventitious agent detection methods

Method	Description	Advantages	Limitations	Limit of detection	Cost[a]	Time	Reference (s)
Growth on agar medium	Supernatant is added to agar medium and allowed to incubate either aerobically or anaerobically for a period	Simple; Accepted by regulatory agencies; Well studied and documented.	Time consuming process; Does not identify the species of the adventitious agent	1 CFU (Sutton, 2011)	$1.50–$2.50[a] per plate	12 hours–14 days	[6,7,63]
Animal inoculation	Animals (such as mice, rats, rabbits, etc.) are inoculated with a small amount of virus containing material and observed for signs of illness	Effective; Accepted by FDA	Ethical concerns; Expensive; Time consuming	21 days; Limited based on observation by researcher (ERSA journal)	$475–$990[b] per test (rabbit pyrogen test)	2−3 hours	[5,8,9]
Hemadsorption test	Culture medium is replaced with a suspension of erythrocytes, and, if the cells are infected with virus, the erythrocytes will adhere to the cells	Well understood	Time consuming, no information on type of viruses/only can be used on certain viruses	Dependent on viral particle and presence of viral hemagglutinin	$34–$75 per test (supplies and labor) (Newton, 2002)	1 hour	[5,62]
Cell culture	Cells are inoculated with a sample that is suspected to be contaminated and observed for cytopathic effects and turbidity.	Simple	Time consuming	1 CFU; Must be estimated for specific experiments (Sutton)	$100–$1 000 000[a] (depends on available equipment and supplies)	2–14 days	[5,6,8–10,63]

[a] Price obtained from Thermo Fisher Scientific, Waltham, MA.
[b] Price from Institute for *In Vitro* Sciences, Gaithersburg, MD.

Table 2

Microscopy methods

Method	Description	Advantages	Limitations	Limit of detection	Cost[a,b]	Time	Reference (s)
Light microscopy	Specimens are observed under light microscope with or without oil immersion	Simple, well understood	No information on species of contaminating agent	200 nm size (Sutton 2011) or 200 000 particles per square millimeter (Forouhi 2020)	$200–$5000[a] (for microscope)	10−30 min. (depending on preparation)	[10–12,64]
Transmission electron microscopy	Specimens are observed via a transmission electron microscope and a high voltage electron beam is used to create an image of the specimen.	Allows for the direct visualization of viruses, can be used with difficult to detect or unknown viruses	Expensive equipment, complex, Time consuming sample preparation	<10 nm (Sutton 2011) (specimens must be prepared) 10E7 pfu/mL	$2 000 000[b] or higher for the microscope, $200[c] per specimen for fixation (off-site), $100–$200[c] for use of microscope (off-site)	3 hours–14 days for fixation, embedding, sectioning, staining, and imaging (on-site or off-site preparation)	[12,13,14••,63]
Immunoelectron microscopy	A sample is suspended in a suitable medium (such as phosphate buffered saline) and antiserum is added. The mixture is warmed, centrifuged, and the pellet is examined by negative stain electron microscopy.	Used extensively for the diagnosis of viral infections (est. 1940s)	Complex sample preparation, previous knowledge of the appropriate antibodies to be used required	10^5–10^7 particles per mL (Li 2013)	$50 000–$1 000 000[b] for microscope $100 for specimen preparation (off-site), $50–$65[c] for specimen mounting and coating (off-site), $100–$200[c] for use of microscope (off-site)	3 hours–14 days for fixation, embedding, sectioning, staining, and imaging (on-site or off-site preparation)	[14••]

[a] Price obtained from Olympus Life Sciences, Waltham, MA.
[b] Price obtained from TSS Microscopy, Hillsboro, OR.
[c] Price obtained from Indiana University, Bloomington, IN.

samples are preconcentrated with membrane filtration or similar methods, this problem may be minimized. This would increase the cost and time of this technique. Table 1 summarizes the historical methods and Table 2 summarizes all the microscopy methods presented herein.

Fluorescence microscopy and other optical methods

Technological developments in recent years have led to the development of portable microscopy [16••]. For example, a smartphone-based fluorescence microscopy method has been developed to enable imaging of various fluorescently labeled objects such as viruses and bacteria [16••]. Shrivastava *et al.* has presented a smartphone fluorescent microscopy-based detection and quantification method for bacteria from liquid samples [17•]. Other smartphone-based detection methods for adventitious agents can be performed based on colorimetric, turbidity, pH, or luminescence-based endpoints [16••]. A unique feature of these methods is that they can be used in real-time. Real-time detection can be implemented using fluorescent measurements or bioluminescence detection and periodical measurements. These smartphone detection methods have been applied in virus and bacteria detection and present a unique platform for adventitious agent detection. Table 3 summarizes these smartphone-based detection methods.

Immunoassays

Immunoassays such as an enzyme-linked immunosorbent assays (ELISA) are a classical method for detecting and identifying adventitious agents [18•,19•]. Recent publications highlight the development of novel immunoassays that present advantages over traditional ELISA. One such method, as presented by Pankratov *et al.*, uses a cellulase-linked immunomagnetic assay for bacterial analysis [18•]. This method, as described by the authors, could detect a single *E. coli* cell which shows the high sensitivity and specificity of the method [18•].

Other immunoassays include cell-based activation immunoassays, lateral flow test strip immunoassays, immunochromatographic assays, and magnetophoretic immunoassays [15,20]. These assays present advantages in detecting adventitious agents. The immunochromatographic assay presented by Li *et al.* was able to specifically detect *E. coli* cells in various sample types [21•]. The cell-based activation immunoassay presented by Bar-Haim *et al.* can detect various bacteria and virus types faster than

Table 3

Smart-phone based and biosensor-based methods

Method	Description	Advantages	Limitations	Limit of detection	Cost[a]	Time	Reference (s)
Smartphone-based fluorescence microscopy	Samples are fixed and stained on glass slides (using a fluorescent-*in-situ*-hybridization approach with pre-designed rRNA-targeting PNA probes) and then are analyzed directly on the smartphone screen. The smartphone is integrated with a 3D printed optomechanical attachment.	Compact, lightweight, cost-effective, simple	Detection limitations, requires PNA probes	10 CFU/mL (Shrivastav [17•])	$2000 or greater[a]	5 min–1 hour (Young-Ho Shin *et al.* 2021)	[17•,60]
Smartphone-based optical methods	Smartphone-based Optical Methods rely on certain endpoints such as pH, turbidity, color, and so on for a measurement to be determined. With each of these methods, something like a smartphone microplate reader or other connected/accessory device may be used to quantify the desired endpoint based on a picture.	Compact, lightweight, portable	Low-throughput, low sensitivity, additional components sometimes required	20 CFU/mL (Nelis, 2020)	$2000 or greater[a]	5 min–1 hour (Young-Ho Shin *et al.* 2021)	[16••,59,60]

[a] Price obtained from ioLight, Hicksville, NY.

traditional ELISA [22••]. Additionally, the lateral flow test strip immunoassays presented by Tominga *et al.* could detect and distinguish 72 distinct types of bacteria [19•]. Finally, the magnetophoretic immunoassay presented by Kim *et al.* could detect the growth of a mycoplasma strain with lower false positives than other methods [20]. The major limitation of ELISA and other immunoassays is the need for prior knowledge of the adventitious agent. Table 4 summarizes the immunoassays presented herein.

PCR methods

Over the last 20 years, polymerase chain reaction (PCR) has been accepted as a gold standard for detecting various nucleic acid-based adventitious agents in pharmaceutical products [23]. Besides traditional PCR, real-time PCR, Droplet Digital PCR (ddPCR), multiplex PCR, and microfluidic PCR have been developed. Real-time PCR assays based on Primer-Probe Energy Transfer (PriProET) have more robust diagnostic capability as they require a shorter conserved region for hybridization making it less susceptible to single point mutations [24–26]. It is suitable for routine screening methods for raw materials, cell banks, viral and vector seeds bank and animal materials. A virus-specific PCR test just before bioreactor harvest has successfully detected and identified a virus contaminant preventing further virus spread and reducing the financial burden of a complete shut down [27,28,29••]. However, prior knowledge of the genome sequence of the virus, bacteria, or mycoplasma is essential to design specific primers [30].

The development of multiplex real-time PCR has enabled differentiation and quantification of viral or bacterial contaminants in a single assay. The risk of carrying-over contaminations is also reduced [31]. It provides increased throughputs and an increase in the number of targets tested in a single reaction by detecting co-infections [32••,33]. This PCR technique is often combined with other methods to detect adventitious agents. Proximity ligation allows for the detection of infectious agents by recognizing an antigen on the viral or bacterial surface with antibodies bound to DNA strands [29••,34]. Also, degenerate oligonucleotide primed (DOP) PCR can be used for non-specific amplification of a DNA sample and is often combined massive parallel sequencing [35••,36]. The digital droplet PCR (ddPCR) is a newly developed PCR technique which enables absolute quantification of target nucleic acids without the need of a standard curve [37•,38•]. High throughput dd-PCR is available but needs better standardization and validation.

In addition to PCR, developments in biosensors have presented a unique technique for the detection of bacteria, mycoplasma, and viruses [39,40]. The Ibis T5000 Universal Biosensor allows for sensitive and specific identification of microbial contaminants [41]. The

Table 4

Immunoassays

Method:	Description	Advantages	Limitations	Limit of detection	Cost[a,b,c,d]	Time	Reference (s)
Cellulase-linked immunomagnetic assay	This assay utilizes a sandwich antibody (Ab/aptamer-bacterium-Ab/aptamer) labelled with cellulase assembled on a micrometer sized magnetic bead applied to a nitrocellulose-modified-film. The cellulase then digests the nitrocellulose film which changes the electrical properties of the electrodes. This change can be measured.	High sensitivity, high specificity	Needs to be assessed for more species	Detection of a Single *E. coli* cell [25]	$515/100 assays[a]	15 min–3 hours (Jamal 2020)	[18•]
Cell-based activation immunoassay	B-lymphocytes or T- lymphocytes or monocytes and granulocytes are used to detect and quantify specimens in samples.	Quick, high specificity	Prior information required, complex	10^5 CFU/mL	$300–$700[d]	24 hours	[22••]
Lateral flow test strip immunoassay/ immuno-chromatographic assay	A liquid moves via capillary action through polymeric strips on which molecules that can interact with the analyte are attached.	Low cost, simple, rapid	Low sensitivity	10^4–10^9 CFU/mL	$110/10 assays[a] $1660/100 tests[b]	15 min (Jamal 2020)	[19•,61]
Magnetophoretic immunoassay	An immunoassay (similar to ELISA) that uses magnetic beads, radioisotopes, or fluorescent labels to detect a specific analyte. A magnetic label is conjugated to either the antibody or antigen and a magnetic reader is used to record the magnetic change induced by the beads.	High specificity, can be fully automated, rapid	Required conjugation of magnetic beads	0.3 pM or 5−50 CFU/mL	$1400 or greater[c]	<3 hours (Jamal 2020)	[20,61]

[a] Price obtained from Thermo Fisher Scientific, Waltham, MA.
[b] Price obtained from Abcam plc., Cambridge, UK.
[c] Price obtained from Tiger Medical, Inc, Irvington, NJ.
[d] Price obtained from RayBiotech, Inc, Peachtree Corners, GA.

technology is based on the coupling of broad-range PCR and electrospray ionization-mass spectrometry (ESI-MS) [40,41]. DNA is amplified using family specific PCR primers targeting organisms of interest. The various nucleic acids that exist in the sample are accurately measured by mass spectrometry and identified utilizing a database of sequence base composition of known micro-organisms [40–42]. This enables broad adventitious agent investigation with PCR primers developed for a wide array of both known and unknown bacterial, mycoplasma, and viral species [43]. However, the method does not determine whether the contaminant is viable or not and thus needs further validation [44,45].

High throughput sequencing (HTS)

High-throughput sequencing (HTS) allows for comprehensive detection for potential microbial contaminants including unknown viruses [46]. HTS detects the presence of any unexpected sequences that exist in a sample via non-specific massive sequencing and identifies the detected sequence by mapping the sequence to an existing database [47]. Multiple studies have proven that the sensitivity of HTS was comparable to that of qPCR assays [48,49]. However, HTS is a complicated technology which involves various upstream sample handling process, different sequencing platforms, bioinformatic analysis tools and databases; therefore, development of control and method standardization is an essential requirement for future HTS to be considered as an ideal detection method [50•].

The sensitivity of HTS is often influenced by genomic size, structure, and relative efficiencies in reverse transcription and cDNA synthesis in the case of RNA viruses [51••,52••]. The difference in sensitivity of viral detection demonstrated in the multicenter study highlights the importance of enhancing sample preparation/processing strategies and the development of reference materials [50•]. Furthermore, infectivity assays need to be combined as the hits identified by HTS analysis does not confirm whether the contaminant is viable or not. A comprehensive standardization in sample preparation, sequencing platform, reference materials, bioinformatics, and databases are critical for the future of adventitious agent detection [53••,54,55].

Finally, a recent rise of Oxford Nanopore's MinION sequencing device has created a paradigm shift. The sequencing is based on the measurement of changes in electrical conductivity generated by different bases as the DNA strand is drawn to a nanopore. This affordable, pocket-sized MinION provides real-time long-read

Table 5

PCR and HTS

Method	Description	Advantages	Limitations	Limit of detection	Cost	Time	Reference (s)
Real time-PCR	Processed sample is amplified with specific primer set and a probe, and Ct value is assessed for identification and compared with standards for semi-quantification in real-time.	Sensitive, specific, quick	No unknown detection, no information on infectivity, semi-quantitative	1–10 copies/μl	$15 000[a]–$80 000[b] $1−5/reaction	30 min–2 hours	[23–25]
Multiplex PCR	With multiple non-interfering primer sets, PCR reactions targeting for multiple sequence are performed within the same well, which are detected by different probes.	Increased throughput, Reduced carrying over contamination	Primer interference, Difficult optimization and validation, semi-quantitative	10−30 copies/μl	$15 000[a]–$80 000[b] Reaction cost varies depending on the number of targets	1−3 hours	[32••,33]
Droplet digital PCR	A nucleic acid sample is partitioned in water-in-oil droplets in which PCR reactions occur. After PCR amplification, the individual fluorescent positive and negative droplets are quantified via Poisson distribution to determine the number of DNA copies in the starting sample.	Sensitive, reproducible, Absolute quantitation, no standard curve required, insensitive to PCR inhibitors	More expensive, more hands-on time, restricted dynamic range	1 copy/μl	Quote requested[c] $3−5/reaction	3 hours	[37•,38•]
High throughput sequencing	Samples are screened for presence of any unexpected sequence via non-specific massive sequencing and the detected sequence is identified by mapping the sequence to the existing database.	large breadth of detection, unknown detection, high throughput	Time consuming, complicated, standardization and reference material establishment, No information on contaminant viability	∼10 copies/reaction	$200 000–400 000[d] $20−1500/GB	10−48 hours	[46–48,50•,51–54]
MinION	sequencer	Target DNA strand is drawn into nanopore, and each DNA base is identified by measuring different electrical conductivities.	Low cost, small size, direct sequencing, real time data collection and analysis, fast	High quality and high copy number required	10 copies/reaction	$1000–$285 455[e] $90−1600/sample	Full: 6−10 hours Quick: 10 min–1 hour	[56–58]
*LOD is inversely	proportional to turnaround time. With high-copy, quick detection is possible						

[a] Price obtained from Qiagen, Hilden, Germany.
[b] Price obtained from Thermo Fisher Scientific, Waltham, MA.
[c] Price obtained from Bio-Rad Laboratories, Hercules, CA.
[d] Price obtained from Illumina, San Diego, CA.
[e] Price obtained from Oxford Nanopore Technologies, Oxford, UK.

Figure 1

	Simple	Rapid	Inexpensive	Unknowns Detectable	Sensitive	Specific	Real Time
Growth on Agar Medium	✓						
Cell Culture	✓						
Light Microscopy	✓						
Handheld Devices	✓		✓				✓
Lateral Flow Strip Immunoassay	✓	✓	✓				
Cell Based Activation Immunoassay		✓				✓	
Magnetophoretic Immunoassay		✓				✓	
Real-time PCR		✓			✓	✓	✓
High Throughput Sequencing		✓		✓	✓	✓	
Transmission Electron Microscopy				✓	✓	✓	
Ibis T5000 Universal Biosensor				✓	✓	✓	
Cellulase-linked Immunomagnetic Assay					✓	✓	
Droplet Digital PCR					✓	✓	
Immunoelectron Microscopy					✓	✓	
Ideal Detection	✓	✓	✓	✓	✓	✓	✓

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Comparison of Detection Techniques: The above chart shows the advantages of each of the detection techniques (red box) described previously along with the pre-processed materials (blue box). The chart shows that no one method has all the characteristics of an 'ideal' detection method. Thus, further development in detection techniques may be able to provide a detection method that is closer to an 'ideal' method.

sequencing allowing for the detection of both known and unknown species [56–58]. With recent improvements in performance and further validation, it could be a viable option for adventitious agent detection where immediate action is required. A summary of the PCR methods, HTS, and biosensors can be found in Tables 4 and 5.

Discussion and conclusions

This review has presented both historical and more novel detection methods. From the presented tables and Figure 1, methods with lower specificity and lower sensitivity tend to have lower costs associated with them whereas more robust, sensitive, and specific methods tend to have higher costs. Thus, it is the burden of the researcher to choose whether to incur the higher costs of specific methods at the price of reduced experimentation or to enjoy more experimentation with the risk of lower sensitivity. Additionally, each method presents wildly different limits of detection. Without clear guidance from regulatory agencies and given the fact that some adventitious agents may not be well understood, it is difficult to know whether a low limit of detection is necessary and worth the cost of some of the more sensitive methods. Thus, continuous efforts in improving detection methods including reducing costs, expanding sensitivity and specificity, and gaining better insight on the requirements by

regulatory authorities is still of paramount importance in the pharmaceutical industry.

Currently, it appears that real-time PCR, high throughput sequencing, and some biosensors are the closest to 'ideal' detection methods and further development of these methods may be the future of adventitious agent detection. The limited capability to detect a broad spectrum of both known and unknown agents by PCR-based method needs to be addressed to become more effective and versatile. High throughput platform development using the combination of family specific primers and specific primer sets can be devised to detect a wide range of agents in a single assay. Moreover, further improvements in performance, speed, affordability, convenience, method standardization, and the establishment of reference materials would make gene sequencing a more attractive approach.

With such a broad variety of detection methods available, it is possible to consider the adaptation of these methods to other uses. As an example, some of the methods presented herein could be adapted for use in the production of gene therapeutics. This transition could lead to better manufacturing techniques and yield significant opportunities for quality by design in gene therapeutic products. Specifically, as gene therapeutics use lentiviral or other virus-type delivery platforms, the use of real-time virus detection methods with quantification could lead to the ability to track the productivity of a gene therapy manufacturing platform. The methods presented previously that could fit this description (with some modification) include real time PCR, real-time immunoassays, and biosensors such as the Ibis T5000 Universal Biosensor. The ability to accurately monitor the production of viral vectors for gene therapeutics could enable for better process decisions and enhanced manufacturing techniques. Thus, the continued development of detection methods for adventitious agents is not only beneficial for safety and quality of pharmaceutical products, but it is also a potential avenue to enhance the manufacturing of future therapeutic products such as gene therapeutics.

Conflict of interest statement

Nothing declared.

Author contributions

Morris and Lee equally contributed to planning, manuscript writing and review. Yoon was the project PI and provided the main supervision and guidance to the research work. All the authors reviewed and approved the manuscript.

CRediT authorship contribution statement

Caitlin Morris: Conceptualization, Planning, Writing and review. **Yongsuk Lee**: Conceptualization, Planning, Writing and review. **Seongkyu Yoon**: Writing editing and review.

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