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REVIEW

Non-conventional expression systems for the production of vaccine proteins and immunotherapeutic molecules

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ABSTRACT

The increasing demand for recombinant vaccine antigens or immunotherapeutic molecules calls into question the universality of current protein expression systems. Vaccine production can require relatively low amounts of expressed materials, but represents an extremely diverse category consisting of different target antigens with marked structural differences. In contrast, monoclonal antibodies, by definition share key molecular characteristics and require a production system capable of very large outputs, which drives the quest for highly efficient and cost-effective systems. In discussing expression systems, the primary assumption is that a universal production platform for vaccines and immunotherapeutics will unlikely exist. This review provides an overview of the evolution of traditional expression systems, including mammalian cells, yeast and *E.coli*, but also alternative systems such as other bacteria than *E. coli*, transgenic animals, insect cells, plants and microalgae, *Tetrahymena thermophila, Leishmania tarentolae*, filamentous fungi, cell free systems, and the incorporation of non-natural amino acids.

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Introduction

During the past few decades, the technology and techniques of recombinant protein expression have been based largely on molecular engineering concepts associated with fundamental aspects of microbiology and cellular biology. One of the major drivers of expression system development has been the ever-increasing demand for large quantities of recombinant monoclonal antibodies, mostly for use in cancer therapy, and the need to produce highly purified and well-defined vaccines against infectious agents, which are sometimes composed exclusively of recombinant viral or bacterial proteins. In parallel with this, molecular biology techniques have gained in sophistication and ease of use, allowing the exploration of novel expression systems. Today, in addition to efficiency, productivity and cost-effectiveness, safety considerations are increasingly demanding, leading to new, stronger regulatory standards in production quality and control, such as the use of alternatives to the traditional antibiotic-based selection system.[1,2]

In the continuously moving field of expression systems, 2 developmental directions should be considered: the rational re-engineering of existing systems and the identification of completely alternative hosts (a strategy which does not, in itself, exclude engineering steps). In the case of re-engineering, the impact of multi-omics or a system-based biological approach may improve the quality of the expressed protein, such as creating a molecule with a better glycosylation profile. In terms of productivity, considering that the physiological limits of any given system cannot be pushed indefinitely, the solution is more likely to come from new hosts with a naturally extended capacity for very high protein production and secretion. In an ideal situation, these alternative hosts would have a fast doubling time, grow in simple and inexpensive media, and be compatible with linear scale-up, creating the capacity for very large production volumes of secreted protein that is easy to purify and with a molecular structure as close as possible to the natural protein.

This review briefly discusses traditional expression systems, such as mammalian cells, yeast and *E. coli*, before examining in more detail several alternative systems, such as non-*E. coli* bacterial systems, transgenic animals, insect cells, plants and microalgae, *Tetrahymena thermophila, Leishmania tarentolae*, filamentous fungi, cell free systems, and the incorporation of non-natural amino acids. The main characteristics of these expression systems are summarized in Table 1.

Mammalian cell lines: Still in pole position?

Mammalian cell lines are used to manufacture diverse immuno- and biotherapeutic molecules, due to their high and robust productivity of secreted proteins in serum-free medium, and their ability to perform complex post-transcriptional modifications.[3] It is also possible for mammalian cell lines to be used for the production of viral vaccines, for example PER.C6, Vero, CAP, AGE1.CR and EB66,[4] but we will focus on 2 commonly used cell lines, the Chinese Hamster Ovary (CHO) and the Human Embryonic Kidney 293 (HEK293) cell lines. Recently these have been stably transfected in order to extend, and possibly to increase, the production of recombinant proteins, and therefore become "non-conventional" expression systems.

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Table 1. Summary of the main characteristics of expression systems for vaccine proteins and immunotherapeutics.

Expression Systems	Speed	Cost	Scale-up capacity	Glycosylation	Contamination risk	Yield for exogenous protein production	Examples of licensed products[3]
Stable non-integrative mammalian cells	Low	High	Medium H	Mammalian glycosylation	Endogenous virus	Low but could be improved	No
Yeast	High	Low	High	Hyper-mannosylation with current yeasts	Low	High	HBV and HPV vaccines
Bacteria other than *E coli*	High	Low	High	None with current bacteria	Endotoxins for Gram-	Potentially high	No
Transgenic animals	Very Low	Very High	NA[1]	Mammalian glycosylation	Endogenous virus	Medium/high	No
Baculovirus/insect cells	Medium	High	Medium	Close to mammals[2]	Baculovirus	High	HPV and influenza vaccines
Silkworm	Medium	Medium	Low	Close to mammals	Low	Medium/high	Feline and canine IFN
Drosophilia S2 insect cells	Medium	High	Medium	Close to mammals	*Copia* virus	Medium	No
Transgenic plant	Low	Low	Medium	Close to mammals	Low	Potentially high	IFN α
Transient plant	Medium	Low	Medium	Close to mammals	Low	Medium	Mab against ebola
Microalgae	High	Low	High	Eukaryotic: Close to mammals Prokaryotic: No	Low	Medium	No
Ciliate (*Tetrahymena thermophila*)	Medium	Medium	Medium	Close to mammals	Low	Medium	No
Trypanosome (*Leishmania tarentolae*)	Medium	High	Medium	Close to mammals	Low	Medium	No
Filamentous fungi	High	Low	High	Close to mammals	Low	Medium	No
Wheat germ-based Cell free	High	High/Medium	Low/Medium	No	Low	Medium	No
***E.coli*-based Cell free**	High	High/Medium	Medium	No	Endotoxins	High	No

[1]Not applicable.
[2]Close to mammals: complex N-linked glycosylation.
[3]Example of licensed products in the field of vaccines and immunotherapeutics.

Chinese hamster ovary

The CHO cell line is the most widely used and most extensively studied mammalian host for the production of monoclonal antibodies; this system has been extensively discussed in various reviews and will not be detailed here.[5] CHO cells growing in suspension in serum-free medium can be used to produce recombinant viral proteins, such as the S and PreS2 proteins of the hepatitis B virus (HBV) surface antigen, which are then assembled into HBV-like particles.[6] Indeed, the GenHevac® B vaccine, which contains these viral proteins, is immunogenic in humans.[7,8] The cytomegalovirus (CMV) gB antigen has also been stably expressed in the CHO cell line, leading to the development of a recombinant vaccine that is immunogenic in humans in presence of MF59 as an adjuvant.[9,10] Recently, the CHO cell line was used by GSK (Glaxo Smith Kline, formerly Novartis Vaccines) to produce a pentameric molecule consisting of the human CMV surface proteins gH/gL/UL128/UL130/UL131A, the pentamer being the main target of neutralizing antibodies in human CMV-seropositive individuals.

To achieve this, the CHO cells were stably transfected with either 2 vectors, one coding for gH/gL, and the other for UL128/130 and 131A, or with a single vector encoding all 5 genes. The level of pentamer production was found to be linked to the vector strategy and to the particular CHO cell lines tested, which included CHO-K1A, CHO-K1B and CHO-DUXB11. The pentamer could be recognized by a panel of conformation-dependent monoclonal antibodies, and induced neutralizing antibodies in mice, suggesting the suitability of using the pentamer as a vaccine in humans.[11]

Recently, Daramola *et al.*, have described a simple, large-scale transfection method using a CHO cell line that stably co-expressed the Epstein-Barr Nuclear Antigen-1 (EBNA-1) and glutamine synthetase proteins.[12,13] The transfection vector consisted of a plasmid containing the origin of replication of the Epstein-Barr virus and encoding the recombinant protein. Although the plasmid was transiently transfected into the recombinant CHO cell line, it was maintained as an episome, with increased and prolonged expression of the protein of interest observed *via* plasmid episomal replication. While recombinant proteins for vaccines or immunotherapeutics have not been expressed using this system, it may be suitable for that purpose. The recombinant CHO cell line expressing the Epstein-Barr protein would, however, need to be approved by the relevant health authorities.

Human embryonic kidney cell line 293

The HEK293 cell line was created by transfection of a human primary embryonic kidney cell culture taken from an aborted embryo with sheared DNA of adenovirus type 5 (AD5).[14] HEK293 is easy to grow in suspension and can be adapted to serum-free medium, although, notably, the tumorigenicity of this cell line is still an issue.[15] The cells are suitable for large scale transient gene expression as they are highly transfectable.[16] Stable expression can also be obtained.

For viral vaccine protein expression, the G-protein from rabies virus assembled in a virus-like particle (VLP) was stably secreted in HEK293 after transduction with a G-protein-expressing lentivirus vector, and these VLPs were immunogenic in mice.[16,17] The HA protein from the A/H5N1 influenza strain, fused with a human IgG Fc tag, has also been stably expressed in HEK293, with the recombinant protein showing biological activity.[18]

Two genetic variants have been described for the HEK293 cell line: the 293E line, expressing, like the CHO cell line described above, the EBNA-1 antigen, and the 293T line, which expresses the Simian Virus 40 large T Ag. These cell lines sustain episomal replication of plasmids containing the EBV and SV40 origins, respectively.[19,20] But, as with the EBNA-1-expressing CHO cell line, the fact that these HEK293 genetic variants constitutively express viral antigens could present challenges for health authority approval.

Mammalian cell lines are still necessary as they are naturally fitted for the production and secretion of complex molecules with precise glycosylation. Their position at center stage is also due to the fact that more than 60% of the currently available immunotherapeutic molecules are monoclonal antibodies. Nevertheless this supremacy is not immutable, and one can easily imagine the emergence of new expression platforms derived from engineered unicellular eukaryote hosts with a high production capacity combined with cost effectiveness.

Yeast: Lessons learned from antiquity

For historical reasons, yeasts occupy a privileged place in the world of biotechnology, particularly within the food industry. Currently, available recombinant vaccines for HBV and human papilloma virus (HPV) are based on 2 antigens expressed in the conventional yeast strain *Saccharomyces cerevisiae*. The first vaccine is formulated with HBV surface antigen (HbS) expressed in *S. cerevisiae* and auto-aggregated as VLPs. This vaccine, developed in 1982 and approved by the United States Food and Drug Administration (FDA) in 1986, has been formulated into 14 monovalent or multivalent vaccines.[21,22] The second example, directed against HPV, is one of the most recently marketed recombinant vaccines. In this case, the antigen expressed in *S. cerevisiae* is the structural L1 protein, a molecule that also auto-aggregates into VLPs. The final formulation of this vaccine contains L1-based VLPs from the 9-valent HPV serotypes mostly associated with the development of cervical cancer.[23]

Recombinant vaccines produced in yeast strains other than *S. cerevisiae* (so-called non-conventional strains) are of great interest for production of vaccine antigens and immunotherapeutics. One such non-conventional strain is *Pichia pastoris*, a yeast originally introduced in the 1960s as an additive for animal foods, and later, thanks to the industrial high cell density fermentation processes already developed for its initial application and the use of strong and tightly regulated promoters, as a new heterologous expression system. Over the years, *P. pastoris* has become a widely used expression system,[24-29] and has been investigated for the production of recombinant protein antigens for human vaccines.[30,31] Several examples of recombinant protein antigen expression in *P. pastoris* show how this system can offer the benefit of higher eukaryotic host cells, with glycosylated and well-folded proteins, while having the advantage of being easy and inexpensive to cultivate.

Efficient secretion of tetraspanin (TSP-2), an antigen from the trematode parasite *Schistosoma mansoni*, is another example based on *P. pastoris* which eventually allowed for the development of a full vaccine manufacturing process for this antigen. Mani *et al.*,[32] showed that the envelope protein from the dengue virus, DENV-2 E, possessed the capacity to form VLPs when produced in *P. pastoris*, and that these VLPs were able to elicit a specific antibody response in mice. In 2002, *P. pastoris* was approved in 2002 by the World Health Organization (WHO) for the production of an HBV vaccine biosimilar by Shantha Biotechnologies (a Sanofi company), and this biosimilar has recently been shown to be potentially more effective than vaccines produced *in S. cerevisiae* in particular patient groups.[33,34]

Hyper-mannosylation, a well-known limitation of conventional yeast systems, is weaker in *P. pastoris*, which has been engineered to produce human-like glycosylation of recombinant proteins by knocking out 4 glycosylation-associated genes and introducing more than 14 heterologous genes.[35,36] In 2004, this technique resulted in the development of Glycoswitch® from Merck, a technology allowing for uniform Man5 N-glycosylation and development of a set of vectors to express glycan-modifying enzymes in yeast strains not limited to *P. pastoris*.[37-39]

Other yeast strains have been explored as expression systems for vaccines or immuno- and biotherapeutic molecules, including *Yarrowia lipolitica, Arxula adeninivorans* and *Kluyveromyces lactis. Yarrowia lipolitica* is a yeast originally developed for oil production, whole cell bioconversion and upgrading of industrial wastes. For the past 30 years, more than 130 proteins, from more than 80 species, have been successfully produced in this yeast.[40] This strain is now being investigated for biopharmaceutical applications due to its modified lipid metabolism pathway. *Arxula adeninivorans* is a dimorphic yeast that can grow as budding yeast as well as mycelium. Wartman *et al.*,[41] showed that this strain has a number of beneficial properties, such as thermo-resistance[42] and halotolerance,[43] which make it an attractive host organism for heterologous gene expression. *Kluyveromyces lactis* is one of the few yeast species that can utilize lactose as a sole source of carbon and energy. It was first used to produce recombinant enzymes for the food industry, the success of which suggests that this yeast could also be used for large-scale therapeutic protein production.[44] Indeed, numerous pharmaceutical proteins have been produced in *K. lactis*, including interleukin-1 β[45] and macrophage colony-stimulating factor.[46] Notably, *K. lactis* is also able to produce single-chain Fv antibodies.[47,48]

New concepts in recombinant yeast vaccine technology are the use of inactivated whole yeast cells,[49] or antigen display at the yeast cell surface.[50,51] These technologies are of great interest for the development of oral vaccines in veterinary medicine using both conventional and non-conventional yeast strains.[52] In 2012, Arnold *et al.*,[53] reported a vaccination approach against infectious bursal disease (IBD) in poultry performed with complete yeast *K. lactis* that expressed defined quantities of the virus capsid-forming protein VP2. For the first time, *K. lactis* was shown to be not only a useful system for the expression of foreign proteins, but also as a vehicle for vaccination.

Bacteria: Between evolution and revolution

Bacterial systems, considered for long time as the workhorse for recombinant protein production have been continuously evolving. With the implementation of new and sophisticated tools they are also entering the era of genomic engineering. A large number of bacterial expression systems have been extensively described and a limited number of them are currently used for the production of recombinant vaccines or immunotherapeutic molecules. It is important to make a clear distinction between systems proposed for laboratory-scale exploration, for example as a commercially available expression kit, and true industrial production, which requires a system suitable for a large-scale fermentation process.

Despite active investigation of novel systems, *Escherichia coli* largely remains the dominant bacterial strain in use. A situation unlikely to change significantly as *E. coli* systems are continuously revisited, being easy to engineer and adapt to new constraints, such as antibiotic-free selection.[1,54] Some alternative gram-negative hosts have been investigated, including *Pseudomonas fluorescens*,[55] for which a complete toolbox is available, although its utility remains marginal compare with *E. coli*. Other hosts, known for their high metabolite output, are currently being explored, including *Ralstonia eutropha*, although with the essential limitation that there are no suitable selection markers, no replicons and more generally no basic genetic elements identified yet for this type of host.[56]

As well as the engineering of novel bacterial systems, development of new bioinformatic software has enabled anticipation of potential expression issues, a critical example of which is protein solubility upon overexpression.[57]

An ideal goal, in terms of metabolic engineering, would be the ability to modify prokaryotic hosts, conferring on them eukaryotic-like characteristics. In 2002, Wacker *et al.*,[58] demonstrated that the N-linked glycosylation process, identified in *Campylobacter jejuni*, can be transferred into *E. coli*. However, while this was a first successful step, the extreme complexity underlying the transfer of a complex eukaryotic biological pathway to a bacterial system cannot be underestimated.[59]

Since the early 1980s, gram-positive bacteria from the *Bacillus* or *Lactococcus* genera have been viewed as potential alternatives to *E. coli* for the production of recombinant proteins, their theoretical advantage being their capacity of secretion and relative adaptation to genetic manipulation. While there is an extensive literature on their use as expression systems,[60] and as live delivery vehicles for mucosal immunization,[61] data supporting their utility remains limited. Further investigation in this field is required.

Transgenic animals: The milky way

Animal transgenesis was developed in the early 1980s, expanding the potential of standard breeding by deliberate engineering of new genetic traits.[62] Since then, techniques allowing precise genomic modification of animals have greatly improved, moving from random genomic insertion to precise site-directed insertions.[63] These new technologies are based on transcription activator-like effector nucleases (TALENs),[64] or the Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR)/

CRISPR-associated (Cas) protein 9 system.[65] A landmark example of the use of transgenic animals as an expression system was the production of human recombinant antithrombin (AT) in goat milk, which was given fast-track approval by the FDA on a compassionate-use basis for 5 patients with hereditary AT deficiency.[66]

Of primary importance is the selection criteria for the transgenic animal bioreactor, which must incorporate several factors, including annual dairy production, reproductive performance, and age of sexual maturity.[67] Besides mammalian species, capable of expressing proteins in milk, transgenic hens[68] or even ostriches[69] can produce proteins in egg albumen. Despite the relative complexity and lengthy timelines associated with the generation of transgenic animal bioreactors, there are some niche therapeutic areas where such a system has utility, including high added-value markets requiring complex molecules for which other systems have failed or which cannot fulfill demand. Such areas include hemophilia A, which is currently treated by repeated injections of expensive factor VIII products.[70]

The production of human recombinant albumin, for which the annual need is estimated to be several tons, may be well served by the use of large transgenic animals, replacing the extraction of the protein from human blood.[71] Gaucher disease, an orphan pathology characterized by the inability of the human glucocerebrosidase enzyme to catalyze the breakdown of glucosylceramide, is a low-volume niche market where transgenic animal-derived treatments may have utility.[72]

While there are numerous examples of therapeutic molecule production in transgenic livestock, development of vaccine antigen production in these systems appears to be a relatively neglected field of study. Nevertheless, the use of transgenic animal systems to produce complex molecules with sophisticated post-translational modification may be useful for a number of potentially problematic antigens. An example of this is the malaria major surface protein (MSP-1) antigen, which has been successfully expressed in transgenic goats for the production of a candidate vaccine.[73]

At the present time, it is difficult to come to any conclusions regarding the utility of transgenic animals as an alternative to other expression systems, or to speculate on potential future developments in this arena. Certainly, as well as continued discussion of the technical aspects of transgenic animal systems, there is an ongoing ethical, and societal debate and safety issues (cross contamination) to be considered.[74,75]

Insect cells and larvae: A profound metamorphosis

Baculoviruses are insect pathogens[76] that can cause fatal disease in lepidopteran, dipteran and hymenopteran larvae, and have, therefore, been exploited as biocontrol agents in agriculture and forestry. The baculovirus-insect cell expression system, often referred to the baculovirus expression vector system (BEVS), is well known today as a protein production platform. The baculovirus double-stranded DNA genome can be easily modified to incorporate genes of interest *via* homologous recombination.[77] Various approaches for generating recombinant baculoviruses have been investigated. The most recently described are an *in vitro* transposition system, adapted from Bac-to-Bac, commercially known as BaculoDirect, and the Flash bac system [78,79] The recombinant baculovirus vectors can then easily be propagated in insect host cells, the commonly used being the Sf21 and Sf9 cell lines from *Spodoptera frugiperda* and the *BTI-TN-5B1-4* cell line (High Five or H5) from *Trichoplusia ni.*

Commercially available vaccines produced in insect cells exist for several different indications in both human and veterinary medicine.[80] In humans, 3 insect-produced vaccines are approved, targeting cervical cancer (Cervarix®; GlaxoSmithKline), prostate cancer (Provenge®; Dendreon), and influenza (Flublok®; Protein Sciences). In animal health, insect-produced vaccines targeting classical swine fever (Porcilis Pesti®; Intervet, and Advasure®; Pfizer) and porcine circo virus 2 (Circumvent PCV2®; Intervet, and IngelVac Circoflex®; Boehringer Ingelheim) have been approved.

Recombinant proteins produced from insect larvae infected with baculovirus expression vectors are also being investigated for commercial use. The first report of the production of human interferon (IFN) α in larvae of the silkworm *Bombyx mori* was published in 1985.[81] Since then, many recombinant proteins have been expressed successfully in silkworms,[82] including recombinant subunit vaccines against influenza and foot-and-mouth disease.[83,84] Recombinant feline IFN omega produced in silkworms (Virbagen® Omega; Virbac) has been approved for feline calicivirus infections in Europe and Canada, while Interdog™, a drug principally composed of canine IFN gamma for the treatment of canine atopic dermatitis, was launched in Japan in December 2005.

Despite these developments, a lack of standard upstream protocols using insect larvae may limit the utility of this expression system in the human health, and as with transgenic animals, the societal debate continues. Interestingly, a flexible Sf9 insect cell line was recently developed leveraging the Recombinase-Mediated Cassette Exchange technology, which is comparable to the baculovirus expression vector system in terms of protein production speed, and has recently been shown to produce complex proteins, such as rotavirus core-like particles.[85]

Drosophilia S2 cells: An alternative approach that can fly

In 1972, Schneider *et al.*, used the late-stage fruit fly, *Drosophila melanogaster*, to derive the Drosophila S2 insect cells. The resultant cell line, Drosophila Schneider line 2, is today used as a stable, nonviral and nonlytic expression system able to successfully produce difficult to express proteins and suitable for cultivation using batch, fed-batch, and continuous cultivation techniques. Indeed, S2 cells have been used over the last 10 y for the clinical development of vaccines, including a subunit vaccine targeting HER2-positive breast cancer, developed by Pharmexa A/S and currently in phase II clinical trials, and vaccines against dengue virus (Merck, Inc.[86]) and West Nile virus (Hawai Biotech[87]) currently in phase I development. Additionally, 2 malaria vaccines, from the Jenner Institute and Copenhagen University, respectively, are soon expected to enter phase I.[88] Drosophila cells, even if still marginal can be considered as a viable or at least complementary option to traditional insect cell lines.

Plant-based systems: Vegetal, but not vegetative

Plant-based systems have recently regained interest after overcoming intrinsic weaknesses, such as the long timeframe associated to their set-up and their GMO status. The high potential of transgenic plants or plant cells as bioreactors is of increasing interest. These represent an alternative, cost-effective eukaryotic system, are compatible with cheap, simple, well-defined and industrially compliant culture media, and provide virological safety. Additionally, even if individual expression yields are somewhat modest, the potentially huge biomass available makes the recovery of recombinant proteins in the kilogram range feasible, although there are intrinsic difficulties in extracting and purifying pharmaceutical-quality proteins from plants, which must not be underestimated.

Plant based-expression systems can be divided into 2 categories, systems using transgenic plants or derived cells obtained through stable genomic insertion, and transient expression systems in which the plant material is only a substrate. A recent review by Takeyema *et al.*,[89] describes in some detail the transgenic technologies behind plant-based vaccines; the current review will therefore only briefly describe these systems.

In transgenic plants, different concepts in system design can be considered, including nuclear genomic insertion and chloroplast transformation. Chloroplast transformation offers the advantage of the multiplicity of organelles involved in expression, although a potential limitation is rudimentary, prokaryote-like profile of the expression pathways.

Protein expression using transgenic plants or derived cells obtained through stable genomic insertion has been successfully demonstrated. The Lex platform recently acquired by Synthon (Nijmegen, the Netherlands), uses a duckweed named *Lemna* as an innovative expression system. Efficacy was demonstrated in a phase I clinical trial of Locteron®, a recombinant IFN α, for the treatment of hepatitis C.[90] Following from the success of Locteron®, a transgenic rice seed-based system for the expression of human lactoferrin was developed by Ventria Bioscience (Junction City, KS, USA), and is now a novel oral agent for the prevention of antibiotic-associated diarrhea. The rice seed-based expression system has also been shown to be suitable for the large-scale production of recombinant human serum transferrin (hTF), with the rice-derived molecule being biochemically, structurally and functionally similar to native hTF, while being produced at a high yield and at a low cost compare with other plasma-derived recombinant forms of hTF.[91]

Recently, BryoTechnology, a next-generation manufacturing system for biopharmaceuticals, has been developed using the moss *Physcomitrella patens*. This system was shown to stably overexpress human α-galactosidase A, with the resultant molecule able to efficiently correct this enzyme deficiency in Fabry mice.[92]

In contrast to the above-describe plant systems, there has been interest in highly efficient transient expression models based on *Agrobacterium* infiltration or similar systems. This new approach offers 2 majors advantages over traditional plant-based systems. From a societal perspective, this approach only uses the plant as a substrate without the need to classify it as a Genetically Modified Organism. Additionally, the production timeline between antigen cloning and large-scale production is very short. This raises the possibility of completely revisiting the production strategy of the annual influenza vaccine, which is currently egg-based, thereby providing an attractive solution to any pandemic outbreak.[93] Indeed, Proficia™ (Medicago), a transient expression manufacturing platform, has been developed and optimized for use with *Nicotiana benthamiana* infiltrated with *Agrobacterium*. Phase II data from its avian flu H5 pandemic vaccine candidate suggest it is safe, well tolerated, and more effective than other vaccines.[94] Currently, the development of a pandemic influenza vaccine is in phase III clinical trials, with emergency use authorization granted in the United States.

Since October 2015, Medicago has been engaged by the Canadian government to develop monoclonal antibodies against the ebola virus. The feasibility of transient expression of 3 humanized monoclonal antibodies against ebola (ZMapp™) using an *Agrobacterium*-mediated *Nicotiana benthamiana* platform has recently been demonstrated by Mapp Biopharmaceuticals,[95] with clinical trials launched in early 2015.[96]

Finally, Elelyso® (Pfizer), a therapeutic recombinant human glucocerebrosidase enzyme taliclucerase alfa produced in carrot cell suspension culture, was approved by the US FDA in 2014 to treat Gaucher disease.[22,97,98]

With regard to vaccines, transgenic plant systems can accommodate the so-called "edible vaccine" concept, an idea that has sparked both interest and controversy regarding both its efficacy and technical considerations including standardization of antigen dose. Nevertheless, the possibilities afforded by edible vaccine candidates in the future, particularly for some applications such as diarrheal diseases, remains of interest, particularly if the plant system takes the form of microalgae.

The use of plant-based expression systems for the production of biotherapeutics and vaccine moieties is potentially a difficult arena, particularly given the long timelines involved with the establishment of transgenic plants, which is hardly compatible with the imposed timeframes of the biotechnology industry. In addition, the mode of cultivation, whether open-field or in greenhouses, is still controversial and subject to negative societal perception, and while plant cells cultivated in a contained environment may overcome these perceptions, they lack the advantages associated with using whole plant systems.

Microalgae: Small cells, big hopes

Algae, close relatives to aquatic plants, are used for the production of a range of compounds used in both the chemical and pharmaceutical industries, including oils, polysaccharides, pigments and pharmacological products.[99]

Marine algae, including prokaryotic marine cyanobacteria, marine eukaryotic microalgae, and seaweed, exist in a range of forms, from individual microscopic cells (*micro*algae) or large plants growing to more than 30 m in length (*macro*algae). Of these, microalgae systems, such as the eukaryotic *Chlamydomonas reinhardtii* and *Dunaliella salina*, 2 marine green algae, and the colorless algae *Schizochytrium sp*, are of most interest, combining high growth rate, ease of cultivation and the ability to perform post-transcriptional and translational modifications of foreign proteins.[100] Indeed, *C. reinhardtti* is the most widely

used microalgae for recombinant protein expression as the cost of production is low and the total process is scalable. Furthermore, several molecular genetic tools are available for this species, including a fully sequenced genome, methods for transformation and mutagenesis, and vectors for secreted or non-secreted recombinant protein production. Stable genetic transformation can be achieved at both the nuclear, chloroplast and mitochondrial genome level.[101]

C. reinhardtti is used for the production of recombinant subunit vaccines, such as the VP1 foot-and-mouth disease protein and the classical swine fever virus E2 protein, both of which can be expressed in the chloroplast.[102,103] In *D. salina,* the HBV surface antigen (HBsAg) has been successfully expressed after transformation at the nuclear genome.[104] The main viral envelope protein of the white spot syndrome virus, VP28, has been expressed in the chloroplast of *C. reinhardtii* and in the nuclear genome of *D. salina*.[105,106] Proteins from the malaria parasite *Plasmodium falciparum*, including the apical membrane antigen 1 (AMA-1) and MSP-1,[107,108] Pfs25 and Pfs28,[109] have also been expressed in *C. reinhardtii*. In fact Pfs25 formulated with different adjuvants induced high-affinity antibodies and block *P. falciparum* infection in mice.[110] *Staphylococcus* and HPV proteins have also been expressed in this system,[107] as well as monoclonal antibodies.[111,112]

Schizochytrium sp. is a robustly fermentable microalgae, characterized by bi-flagellate zoospores and filopodia-like extensions of the cytoplasm.[113] It is used for large-scale production of docosahexaenoic acid (DHA) as well as a nutritional food supplement.[114] Following the genome sequencing of this organism, genomic tools were developed to enable the transformation by different DNA vectors.[115] Using this expression system, the full-length membrane-bound haemagglutinin glycoprotein of 2 H1 influenza strains, H5 and B, were successfully secreted in a form suitable for vaccine formulation. The resulting purified protein from the A/PR/8/34 (H1N1) strain was found in mice to be immunogenic and protective against a lethal challenge with homologous virus.[116]

Subunit recombinant vaccines expressed in microalgae are candidates of choice for oral administration,[117] the system allowing for production of complex antigens that elicit immunogenic responses, while numerous reports have identified algae-derived compounds as immunomodulatory molecules.[107] For example, the D2 fibronectin-binding domain of *Staphylococcus*, fused to the cholera toxin B and expressed from the chloroplast of *C. reinhardtii*, was shown to be immunogenic in mice after oral administration of lyophilized alga powder.[118] The algae vaccine being stable for more than 1.5 y at room temperature. The chimeric protein comprising the C-terminal domain from the AMA1 or MSP1 fused to the algal granule-bound starch synthetase expressed from the chloroplast of *C. reinhardtii* was also found to be immunogenic in mice fed with the starch particles combined with the B subunit of the heat-labile *E. coli* enterotoxin.[108]

Ciliate tetrahymena thermophila: A multi-faceted organism

The sustained and increasing demand for new recombinant proteins has driven interest in alternative and "non-conventional" expression hosts, such as *Leishmania tarentolae* or *Tetrahymena thermophila*. The rationale for investigating such concepts is the need for high productivity at an affordable cost, although there may be major regulatory issues associated with these new hosts.

One emblematic example of this category of expression systems is the non-pathogenic ciliate *Tetrahymena thermophila*. Before consideration as an expression system, it was in *T. thermophila* that catalytic RNAs (ribozymes) were first discovered.[119] Since their characterization, ribozymes have been extensively proposed as new site-specific therapeutic effectors, and also positioned as a key factor supporting theories on the origin of life.[120]

T. thermophila is one of the best-characterized unicellular eukaryotes with the potential utility as a high-quality expression system, with high bioreactor cell densities achievable ($> 2 \times 10^6$ cells/mL) in short generation times. An active form of the human enzyme DNaseI has been expressed in *T. thermophila,* and disulfide bridges were also correctly formed with a consistent glycosylation pattern.[121] Another example is the production of correctly folded *P. falciparum* proteins for use as vaccines, including a synthetic vaccine antigen composed of the N- and C-terminal regions of MSP-1 which was successfully secreted into culture medium and shown to be immunogenic in an MF1 mouse model.[122] Previously, the stable expression of full-length *P. falciparum* circumsporozoite protein was reported, with the expressed antigen containing the natural secretory signal sequence and the C-terminal glycophosphatidylinositol (GPI) anchor, as shown by immunofluorescence imaging.[123]

Overall, the *T. thermophila* system, while still at an early stage of development, has already shown great potential. Further developments are ongoing, such as the engineering of a codon-optimized green fluorescence protein that can be used for localization and visual traceability of the target protein *in vivo* and *in vitro* during production.[124] Additional benefits of this system include the ability of ciliate mitochondria to release their contents to the extracellular space either in a soluble form, or in association with membrane vesicles at the cell periphery, raising the possibility of displaying GPI-anchored surface antigens.[125]

Trypanosome (Leishmania tarentolae): The positive side of a parasite

Leishmania tarentolae, a non-pathogenic trypanosomatid protozoan parasite of the white-spotted wall gecko (*Tarentola annularis*), has been used to express various recombinant proteins with oligosaccharide structures with N-linked galactose and fucose residues close to those of mammals.[126,127] *L. tarentolae* is readily propagated in suspension to high cell density ($> 10^8$ cells/mL) in a simple incubator at 26°C with liquid media containing animal components that can be replaced by recombinant nutriments. Heterologous proteins can be produced intracellulary or secreted into the medium, allowing an easy purification of the recombinant product.

L. tarentolae can be efficiently and stably transfected by various types of genetic vectors. Two main expression systems have been used for protein expression, the constitutive expression system using RNA polymerase I-mediated transcription in the rRNA locus, into which an expression cassette is inserted,[128]

and the inducible expression system, using a transgenic strain of *L. tarentolae* that expresses T7 RNA polymerase and the tetracycline repressor. The tetracycline-regulated expression cassette is integrated into the genome, and expression of the target gene is induced by the addition of tetracycline to the culture medium.[129] A new type of expression methodology based on linear elements has been developed, where linear episomes with telomere-like structures are transfected into the cells, allowing formation of a culture overexpressing the recombinant protein of interest, without clonal selection of the transfected cells.[130]

Proteins produced in *L. tarentolae* include the recombinant human coagulation factor VII,[131] and the glycosylated soluble amyloid precursor protein α (sAPPα), a cleavage product of the etiologic agent in Alzheimer disease, APP.[132] Another study produced the ORF2 coding for a truncated capsid protein of porcine hepatitis E virus, in order to develop an ELISA.[133] Indeed, *L. tarentolae* could also be used to produce monoclonal antibodies, but at a lower yield than CHO, for example, as the process has not yet being optimized.[134]

In the present authors' laboratory, the inducible expression system of *L. tarentolae* was used to produce recombinant haemagglutinin proteins from 6 influenza strains, among these a 2009 A/H1N1 pandemic strain from swine origin, A/California/07/09(H1N1). Soluble, glycosylated haemagglutinin proteins were secreted into the cell culture medium, were easily purified, and found to be immunogenic in mice at a dose of 10μg when administered twice with an oil-in-water emulsion-based adjuvant.[135]

Overall, these data indicate that the *L. tarentolae* expression system may have potential as an alternative or complementary system to the current egg-based influenza vaccine production system, and could also be used to produce other bacterial and viral proteins.

Filamentous fungi: Secretion as a way of life

Filamentous fungi, multicellular eukaryotic microbes used to produce endogenous proteins such as hydrolytic enzymes used in the food, textile, paper and chemical industries, are now increasingly being used to produce heterogeneous proteins. The most attractive feature of filamentous fungi is related to their natural capacity of secreting very high amounts of enzymes. Compared to mammalian cell lines such as CHO, fungal systems provide faster and less expensive fermentation.[136]

Aspergillus and *Trichoderma* are commonly used species. *Aspergilli* are a large and diverse genus of filamentous fungi, with large genomes including many genes homologous to those found in higher organisms. They are especially promising as a host for recombinant protein production as, like mammalian cells, they can perform post-translational modification, but can be cultivated as easily as prokaryotes.[137] For example, human IFN α 2 was expressed in *A. nidulans* by fusing the cDNA of a fungal promoter with a synthetic signal sequence,[138] while interleukin-6 was produced in *A. niger*, after fusion with native glucoamylase allowing its secretion.[139] Monoclonal antibodies can also be produced in *Aspergillus*,[140] but so far no viral proteins have been produced in this system. Of note, several *Aspergillus* proteins have intrinsic antiviral activity, such as the various *A. terreus* antivirals potent against herpes simplex.[141]

Trichoderma reesei is a filamentous fungus first isolated in the Solomon Islands during the Second World War,[142] and has been used extensively to produce cellulases and hemicellulases at a yield of 40 g/L culture medium, representing a very high production and secretion capacity in well-defined fermentation conditions. Transformation occurs through stable integration into the genome, resulting in transformants with different copy numbers and integration sites. The most frequently used promoter for protein production is *cbh1*. While production of heterologous proteins has been moderately successful,[143] and fragment antigen-binding (Fab) molecules have been produced,[142] viral proteins have not.

Another interesting filamentous fungus is *Myceliophtora thermophila,* previously known as *Chrysosporium lucknowense* C1,[136] first isolated from forest alkaline soil in eastern Russia as secretor of neutral cellulase. It shows low viscosity morphology, allowing easy, large-scale, high-cell-density growth in fermenters using inexpensive substrates. Furthermore, this fungus supports broad ranges of pH (4.5–7.0) and temperature (25–44°C) for growth. A C1 genetic toolbox is available, and the 3eight megabase pair genome has been sequenced, enabling the development of various gene expression strategies. One drawback of this system is the production of proteases that can destroy the expressed protein product, although strains have been developed with reduced proteolytic activity through random mutagenesis. High cellulase (HC) and low cellulase (LC) strains have also been developed, with LC strains expressing much less homologous protein than the HC strain, but retaining the capacity to secrete a significant amount of enzyme. Strong gene promoters for the HC and LC strains include *cbh1* and *chi1*, respectively, and the expression of heterologous proteins at high level is facilitated by random integration of one to 10 copies of vectors into the C1 genome. This has allowed, for example, expression at the g/L level of a biologically active heterologous full-length human antibody anti-tumor necrosis factor α, using a glucoamylase carrier to allow efficient secretion. Recently, new genetic tools have been developed using a target gene disruption approach mediated by *A. tumefaciens*.[144]

Cell-free translation: A dizzying scaling-up

The first reported *in vitro* artificial incorporation of carboxy radio-labeled amino acids into proteins from rat liver cells extracts took place in 1948,[145] and more generally, since the early 1950s,[146] it has been shown that disrupted cells from both eukaryotes[147] or prokaryotes[148,149] are still capable of synthesizing proteins. Based on these observations, "cell-free translation" systems have been developed and used to obtain minute amounts of labeled proteins for identification or characterization in the laboratory setting.

In principle, it is possible to prepare a cell-free extract for *in vitro* translation of mRNA from any type of cells, including the yeast *Saccharomyces cervisiae,*[150,151] or tobacco cell lysate.[152] In practice, however, only a few cell-free systems have been developed for *in vitro* protein synthesis. In general, these systems are derived from cells engaged in a high rate of protein synthesis, and commercially available systems exist for various applications including ribosome display and unnatural amino acid incorporation.[146]

The development of cell-free systems depends upon both qualitative and quantitative considerations. Qualitative control may be achieved through use of insect cells,[153,154] or HeLa cells,[155] to overcome improper protein folding or glycosylation. The quantitative aspect of these systems, however, remains the production bottleneck. Nevertheless, the productivity of cell-free translation systems has improved greatly over the last 2 decades. An important step was to move from qualitative to semi-quantitative or even quantitative use of the technique and to further define the so-called "continuous-flow cell-free" translation[156] and "continuous-exchange cell-free" translation.[157] These systems, often using *E. coli*-derived extracts, are able to produce milligram amounts of a desired product, and are suitable for automation. Recent improvements include the possibility of expressing proteins with disulfide bonds through co-translation or addition of disulfide isomerase and/or chaperones to assist with protein folding.[158,159] The increasing interest in such production techniques has driven the development of reconstituted cell-free RNA and protein synthesis systems using recombinant elements.[160] The recombinant nature of the various components allows for an increase in productivity and global efficiency, as elongation factors, ribosome recycling factors, and release factors can be individually adjusted.[161]

In the future, it is possible that cell-free translation systems may achieve a high quantitative mode of protein production, and certainly these systems will be of particular interest in the production of recombinant vaccines.

The potential for scale-up of cell-free protein production systems has recently been demonstrated in the production of milligram-range products using wheat germ extracts in combination with an automated device (CellFree Sciences),[162] which may eventually lead to gram or kilogram-range production with *E. coli*-based open cell-free synthesis (OCFS; Sutro Biopharma).[163] The OCFS system has also been used to identify chaperone proteins that improve the folding and assembly of the monoclonal antibody trastuzumab (Herceptin®; Genentech), a targeted therapy for the treatment of HER2-positive metastatic breast and gastric cancers. In the OCFS system, the disulfide isomerase DsbC and the prolyl isomerase FkpA were identified during screening as important positive effectors for a correct folding of proteins.[164] This lead to a systematic re-examination of the optimal concentration of the essential components of the cell-free protein synthesis reaction mixture, which resulted in a 95% reduction in reagent costs.[165] These results, while obtained using an antibody as a model, are likely to be applicable to other recombinant immunotherapeutic molecules and vaccine antigens.

In addition, cell-free translation has demonstrated a high potential in high-throughput protocols, in which several hundreds of molecules could be expressed per day without prior cloning with rapid and efficient purification processes.[166] Using this strategy, 30 potential vaccine candidate proteins from *Mannheimia haemolytica* were produced in an *E. coli* cell-free system,[167] and 27 pre-erythrocytic antigens from *P. falciparum* were expressed in a wheat germ cell-free protein expression.[168] The system, adaptable to any target, may be a feasible alternative to the standard and time-consuming cloning, expression and screening procedures.

Incorporation of non-natural amino-acids: Revisiting genetics and protein synthesis

Cell-free expression opens the path to the synthesis of unexpected new molecules, such as polypeptides containing non-natural amino-acids. For example, modified amino-acids artificially linked to suppressor tRNAs can be incorporated, through recognition of amber codons, at specific sites within a sequence.[169,170] The ability to incorporate unnatural amino-acids into a given protein in a site-specific manner opens a wide array of opportunities, including easy incorporation of biomarkers, fluorescent or radio-labels for both research and diagnostic applications, and access to new categories of sequence-defined biopolymers.[171]

Some of these applications are more directly applicable to vaccines,[172] including site-specific conjugation of polysaccharide structures, addition of immunogenic amino acids to break tolerance and generate vaccines targeted against autologous proteins associated with cancer, the ability to achieve modifications such as lipidation which are otherwise difficult to carry out in standard conditions, and the ability to mimic eukaryotic post-translational modifications in prokaryotic bacterial hosts. Of note, non-natural amino acids could play a role as adjuvants, knowing that, for example, lipidation is a key factor of the immunogenicity of some vaccine antigens.[173]

Concluding remarks and perspectives

While expression systems for the production of next generation vaccine proteins and immunotherapeutic molecules are reaching maturity, this does not exclude the possibility of breakthrough innovations in the future. This dynamic field of investigation is continuously challenged by the need for rapid, efficient, robust, safe and cost-effective solutions to fulfill an ever-increasing demand, although the quest for an ideal and universal host vector combination is likely to be endless, and every protein is unique, and would require a dedicated strategy ensuring its optimal expression and biological activity.

The prominent impact of multi-omic approaches and molecular engineering techniques allowing direct intervention in the genome of expression hosts will allow fine-tuning of existing systems, allowing their full potential to be exploited. In parallel, the development of cell-free synthesis on a larger scale, with reliable incorporation of non-natural chemical structures, will open the pathway toward new options to address urgent medical needs.

Abbreviations

AD5	adenovirus type 5
AMA	apical major antigen
APP	amyloid precursor protein
AT	antithrombin
BEVS	baculovirus expression vector system
BPI	glycophosphatidylinositol
CHO	Chinese hamster ovary
CMV	cytomegalovirus
CRISPR	clustered regularly interspaced short palindromic repeat
DHA	docosahexaenoic acid

EBNA	epstein-barr nuclear antigen
FDA	food and drug administration
GPI	glycophosphatidylinositol
HBV	hepatitis B virus
HC	high cellulase
HEK293	human embryonic kidney
HPV	human papilloma virus
hTF	human transferrin
IBD	infectious bursal disease
IFN	interferon
LC	low cellulase
MSP	major surface protein
OCFS	open cell-free synthesis
TALENs	transcription activator-like effector nucleases
TSP	tetraspanin
VLPs	virus like particles
WHO	world health organization

Disclosure of Potential Conflicts of Interest

Authors are employees of Sanofi Pasteur or Bioaster.

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