Creating VACCINES drop by drop

The recent outbreak of COVID-19 has highlighted the need for fast and efficient development and production of vaccines for a whole gamut of pathogens, old and new. Microfluidics technologies can be used to accelerate the vaccine development process in a number of ways, not least in the directed evolution of the strains and host organisms used in the production of vaccines based on recombinant DNA. There are also significant advantages to using this powerful droplet technology in the formulation of adjuvants and, of course, in the encapsulation of the vaccine particles themselves, with the potential to scale up production to volumes that equate to billions of vaccine doses. In this article we give an overview of the recent fast-moving advances in vaccine production and demonstrate how microfluidics is proving invaluable in the urgent crisis facing the world today.

By Richard Gray

Vaccination plays a central role in managing many diseases and has been widely used globally since Edward Jenner and a number of his contemporaries successfully demonstrated inoculation against smallpox in the late 18th century. Vaccination works on the principle that introduction into a healthy individual of a biological preparation representative of a disease-causing micro-organism leads to that individual producing antibodies against that organism. In the late 1700s, cowpox was used as a proxy for smallpox, as this is a similar, but far less virulent, virus causing milder symptoms. This approach was quickly superseded by using a dead (whole inactivated) or weakened (live attenuated) form of the micro-organism, reducing the risk of vaccination leading to infection.

A more recent development is the use of recombinant or synthetic DNA to produce a biomolecule representing just a single toxin or surface antigen from the pathogenic organism. This precise, targeted approach ensures a more consistent immune response and level of protection, and can be further enhanced using a variety of strategies designed to present the antigens in such a way, or in combination with other molecules, so as to promote the strongest possible immune response. The exact method or combination is dependent on the type of pathogen (bacteria, virus, etc), its mutation rate, and how it affects an individual's immune system.

New approaches to viral vaccine development

The development of effective vaccines against viruses is particularly challenging due to a combination of the small number of potential target antigens – for example, the SARS-CoV-2 genome encodes just 29 proteins, compared to more than

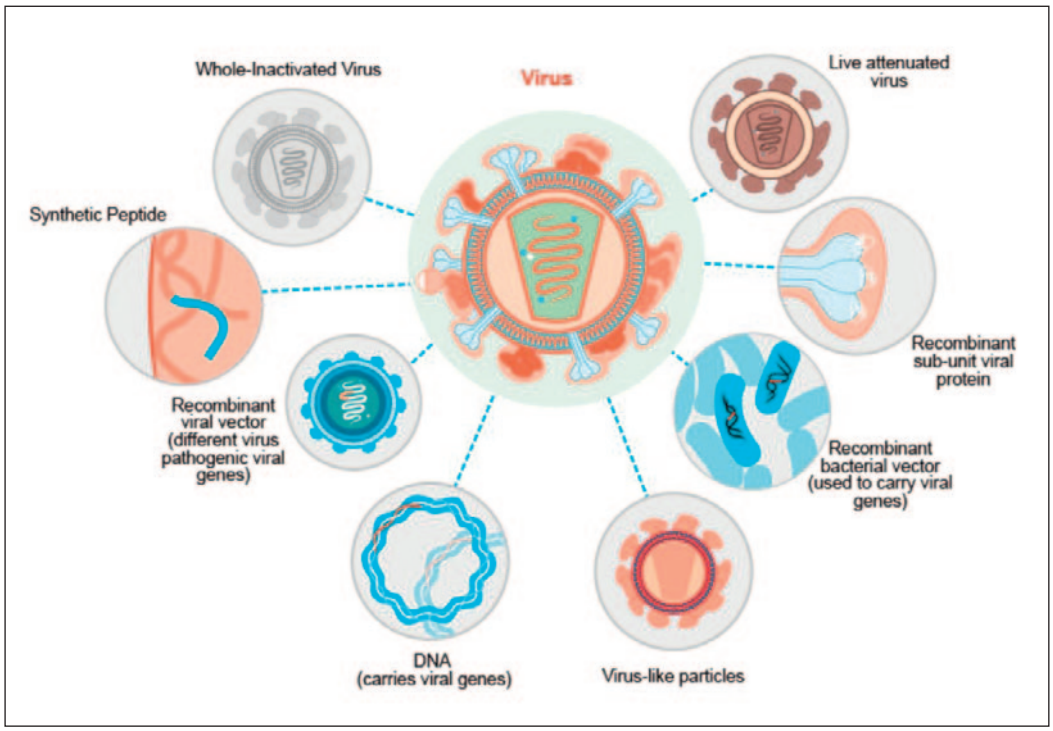

Figure 1
Schematic representation summarising multiple approaches used for the development of vaccines against viral pathogens

20,000 protein-coding genes in humans – and the often high rate of mutation. This combination of factors makes it far more difficult to select an antigen that will give rise to the required immune response and also offer long-term protection.

There are various strategies for the development of vaccines against viral pathogens (**Figure 1**). These include the two traditional approaches – using either whole inactivated or live attenuated viruses – as well as a number of molecular engineering techniques, which are briefly outlined below.

Synthetic peptides

Although most viral proteins are hundreds of amino acids long, the antigen recognised by the immune system is often a relatively short sequence or peptide. It is therefore possible that a completely synthetic peptide can be used to mimic this immunogenic sequence and generate the desired immunoprotective response. The advantages of this strategy are that there is very little chance of the short sequence undergoing mutation or revision, and pathogenic organisms or toxic substances are not required for production. There is also the potential to chemically alter the synthetic peptide to improve stability or reduce off-target effects. The downside of this approach is that many antigens are not simple linear amino acid sequences, and are dependent on complex three-dimensional folding of the viral proteins, requiring laborious modelling of tertiary protein structures to identify the immunogenic epitope.

Recombinant viral vectors

Inserting the genetic material encoding the antigenic proteins from one virus (the target) into a different virus (the vector) offers a potential route for generating an immune response without the risk of infection from the target. This approach often uses attenuated forms of a different, sometimes pathogenic, virus that has had a portion of its own genome deleted to prevent infection. Host cells infected (transduced) by the recombinant virus then produce the antigenic protein, leading to immunorecognition and immunisation.

Recombinant bacterial vectors

Similar to recombinant viral vectors, a number of bacteria, such as *Salmonella*, *E. coli* or *Mycobacterium*, can be used to introduce genes from the target virus into a host. As with viral vectors, a weakened form of the bacteria is generally used to reduce the risk of a bacterial infection, while still allowing intracellular expression of the target antigen.

Plasmid DNA vaccines

A more recent, and direct, approach is to use a plasmid for direct introduction (transfection) of

the antigen-producing gene into the host. The host cells then produce the antigenic peptide or protein in the absence of an infectious agent. This offers similar benefits to synthetic peptide vaccines in terms of vaccine stability and ease of production, and can be combined with adjuncts (to aid cell entry) and/or adjuvants (to stimulate a stronger immune response).

Virus-like particles (VLPs)

VLPs closely resemble viruses, but do not contain any viral genetic material, so are not infectious. They are often produced through *in vitro* expression of viral structural proteins, which then self assemble to create a three-dimensional structure that mimics the target virus. VLPs act in a similar manner to whole inactivated or live attenuated viruses, and have been shown to generate a stronger immune response in some cases. They also have the benefit of being faster and easier to produce than these traditional vaccines, without the risk of infection.

Recombinant sub-unit viral proteins

These vaccines rely on the same technologies and immune response as recombinant viral or bacterial vector vaccines, but instead of inserting a complete viral gene into the vector, only the genetic material of a single subunit of the protein is used. The advantages of this strategy include easier genetic modification and a reduced risk that mutations in the target pathogen will render the vaccine ineffectual, but requires more specific knowledge of the structure of the antigenic epitope and viral mutation rates to ensure effective protection.

A role for microfluidics

Molecular engineering approaches to vaccine development offer a variety of advantages over the use of inactivated or attenuated viruses, with the exact benefits dependent on the method and pathogen of interest. However, the main challenges with all the modern vaccine development approaches is that they rely on knowledge of the genetic sequence of the pathogenic virus – although this is less of an issue with the low cost and high throughput of current next generation sequencing technologies – and identification of the antigenic epitopes that lead to immunisation. And once the correct epitopes have been identified, the relevant genetic elements still need isolating and inserting into the appropriate vector or bioproduction organism.

In each case, this process is a numbers game, requiring hundreds or thousands of experiments to determine and isolate the most appropriate viral antigen, insert the relevant genetic sequence into a vector or bioproduction micro-organism and establish the optimal vaccine formulation. Many of these experiments will also require the assessment of tens of thousands of individual cells, making techniques for effective, high throughput analysis essential. Microfluidics can be a powerful tool to aid and accelerate vaccine development, providing a convenient solution for everything from single cell encapsulation for sequencing or strain evaluation to high throughput generation of adjuvants and vaccine delivery particles.

Single cell RNA sequencing

Single cell RNA sequencing (scRNA-Seq) is increasingly being recognised as an essential tool to improve our fundamental understanding of tissues at the cellular level, as well as how the immune system interacts with pathogens to clear infections and develop immunity. The ability to isolate and study individual cells has led to a number of important discoveries, such as the true diversity of dendritic cells and the identification of a number of novel T cell regulators. It is also a vital aid to vaccine development, enabling the rapid identification of viral genetic material *in situ* within an infected cell to aid antigen selection.

scRNA-Seq is reliant on obtaining the transcriptome of thousands of single cells in isolation, and is commonly performed using a 'drop-seq' protocol, where tens of thousands of single cells are individually encapsulated with uniquely barcoded mRNA-capture beads. This captured mRNA then undergoes reverse transcription (RT-)PCR to generate a cDNA library ready for sequencing. Microfluidics has become an enabling technology in this field as, unlike traditional approaches, it offers an accurate, reproducible and automatable method for efficient encapsulation of individual cells in a high throughput format. The introduction of highly-automated, commercially-available microfluidics set-ups designed specifically for this application has opened up this technique to a wide range of labs, offering straightforward encapsulation without the need for in-house microfluidics expertise.

Recombinant nucleic acid vaccines

Nucleic acid recombination – transposing RNA or DNA from one organism into another – is now routinely performed in thousands of labs around the world, using a variety of methods. However, regardless of the technique used, this approach

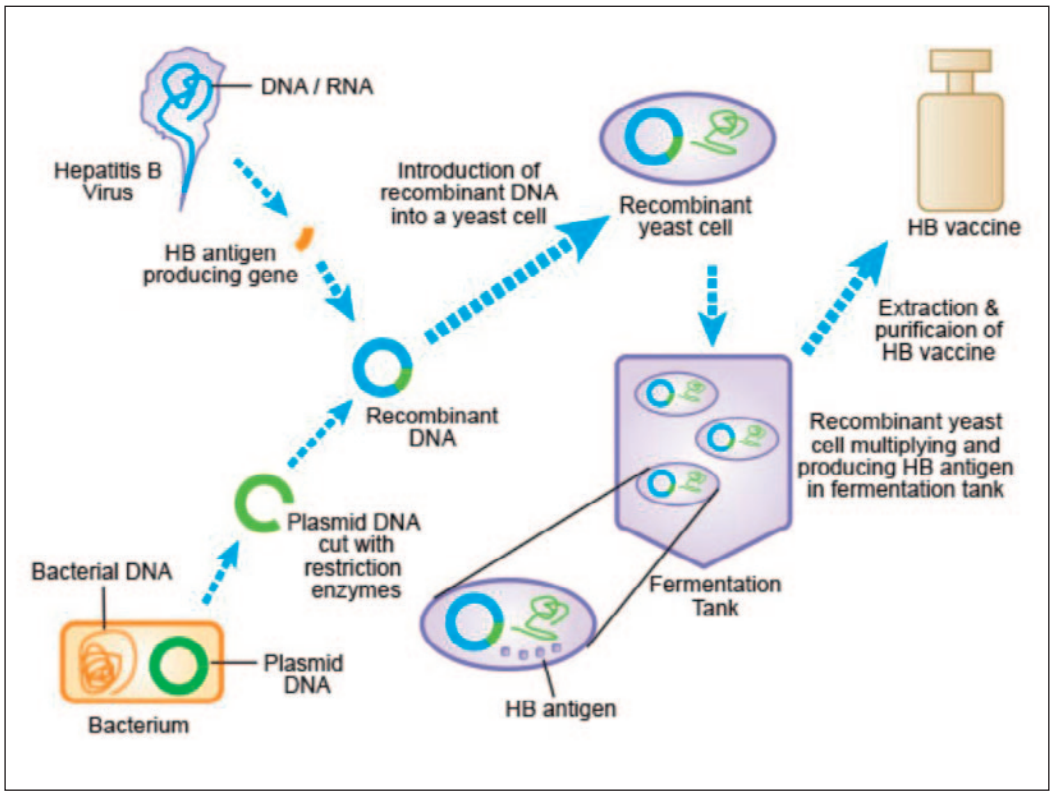

Figure 2
Schematic representation of hepatitis B (HB) vaccine production. An antigen-producing gene isolated from the HB virus is inserted into a bacterial plasmid vector. A yeast cell is then transformed using this vector and grown in a fermentation tank to produce HB antigens, which are subsequently isolated and purified to produce the HB vaccine

relies on attempting to insert the novel genetic material into tens or hundreds of thousands – or even millions – of individual organisms simultaneously. The resulting genetically-modified organisms (GMOs) then need to be screened to determine the successful insertion and expression of the target sequence.

Flow cytometry and fluorescence activated cell sorting (FACS) have become popular approaches for this type of screening, allowing rapid assessment and sorting of individual cells. While not essential, particularly for lower throughput applications, encapsulation of cells in microparticles provides a number of benefits for flow cytometry: the microparticles are mechanically stable, reducing cell shearing and allowing higher flow rates; co-encapsulation of cells with fluorescent reagents allows a broader range of parameters to be assayed; and consistent individual encapsulation and particle sizing reduce background 'noise' and simplify analysis for large data sets. Recent advances in microfluidics have made this approach far more accessible, and there are now a number of encapsulation systems on the market that allow the easy, automated generation of both single and double emulsion particles to suit a variety of workflows (eg Encapsulator, Dolomite Microfluidics).

Viral antigen production

The generation of viral/synthetic peptides, VLPs and viral protein sub-units for direct stimulation of immune responses requires similar genetic engineering approaches to the production recombinant nucleic acid vaccines, albeit with an amino acid rather than a nucleic acid end product. Biomanufacturing workflows – such as the one depicted in **Figure 2** – are often highly complex and difficult to scale up, requiring careful clone selection and process optimisation to achieve efficient expression of the desired final protein. Microfluidic encapsulation of individual bacterial or eukaryotic cells is therefore an important tool for biomanufacturing, helping to improve the efficacy of screening activities at various stages of process development.

Delivery particle generation

Once the desired immunogenic agent has been generated, it still needs to be delivered into the body in a manner that will result in a sufficiently strong immune response to provide protection from the target viral pathogen. Encapsulating a vaccine or therapeutic drug in a nano- or microparticle is an increasingly popular method for drug delivery, helping to overcome stability, toxicity and solubility issues to improve pharmacokinetics and phar-

macodynamics. For example, the free immunogenic agent may simply be too unstable to be transported and stored without degradation, or may be metabolised too quickly on entry into the body, before it can stimulate a sufficient immune response to provide long-term protection.

There are various types of particle in development for drug and vaccine encapsulation – such as biodegradable polymers and liposomes – and the exact formulation of the particle can affect the degree of protection it offers, the rate of release and adsorption, and even the site of release, by binding proteins or antibodies to the external surface of the particle to target a specific cell type. The exact size and composition of the particle, as well as the efficiency of encapsulation, is therefore crucial. The speed, reproducibility and scalability of microfluidics is ideally suited to vaccine encapsulation, offering the ability to easily and reliably adjust particle formulations during development, as well as offering a straightforward route to large-scale production (Telos, Dolomite Microfluidics).

Adjuvant formulation

Presenting an immunogenic agent to the immune system in the correct conformation is not always enough to generate the strength of immune response necessary to provide immunity. In these instances, an adjuvant is often administered alongside the target antigen. Adjuvants do not confer any immunity directly, but act in some way to stimulate the immune system's response to the target antigen. This can be by extending the amount of time the antigen is present in the body, increasing its uptake by antigen-presenting cells, activating macrophages and lymphocytes, supporting the production of cytokines, or acting as an irritant to amplify the overall immune response.

The most common adjuvant in use today is aluminium hydroxide, which has been used for more than 70 years, but a new generation of oil and emulsion adjuvants that offer more targeted stimulation of specific immune response elements are now being developed. Many of these new adjuvants are administered as nano- or microparticle droplets in suspension with the target antigen, and are being produced using microfluidic techniques. For example, a number of saponin-based adjuvants have been recently reported, with the saponin's exact formula and length of saccharide chain determining the efficacy in combination with a given antigen. The consistency and fine control of flow rates provided by microfluidics make it a valuable tool in the development of saponin-based adjuvants, allowing multiple reagents to be combined in precise ratios to allow the generation of large libraries of compounds for screening, as well as offering a scalable method for high throughput production. Saponins are also a key component in another interesting class of novel adjuvants – immune-stimulating complexes (ISCOMs). These are spherical structures, typically around 40nm in diameter, that form spontaneously when certain plant-derived saponins are mixed with cholesterol and phospholipids under specific conditions. The resulting cage-like complex has immune stimulating properties, helping to induce a stronger immune response and longer protection.

Summary

The various examples discussed here demonstrate the broad applicability of microfluidic technologies in vaccine development and production. The reproducibility, scalability and precision offered by the advanced instrumentation now available opens up a diverse range of applications that may not have previously employed microfluidics, helping to simplify analysis and accelerate research. This trend is set to continue, as more labs take advantage of the superior encapsulation efficiency, particle generation and reaction control microfluidics offers. DDW

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