The Age of Phage

A new surge of interest in bacteriophages has led to revived investment in this niche research area despite a history of failed commercialisation. But how can today's techniques overcome yesterday's mistakes?

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Bacteriophages, also known as phages, are viruses that infect bacteria but are unable to infect higher organisms (see Figure 1). They are normal components of every environment containing bacteria, including soil, fresh and sea water, and sediments. They are also present in animals, including humans, where they are an important component of different microbial communities including gut microflora. Although they are subject to phagocytosis and some of them posses motifs, which allow them to bind to integrins, they are safe for humans and animals (1,2). In biopharma, they are usually considered a dangerous contaminant in both laboratories and in bacteria-based production facilities, as they can paralyse the productivity of any facility and, once spread, are usually hard to eradicate (3). However, they also offer many potential benefits due to a few features that are the result of their make up, which have been re-discovered recently. After decades of being neglected by the vast majority of the scientific community, in the last decade they were the basis for setting up several companies.

Why are Bacteriophages Attractive to Biopharma?

Bacteriophages kill bacteria and are potentially helpful in replacing or augmenting antibiotics actions. In order to kill bacteria, they must first attach to them, which requires the detection of specific structures on their surface. This property can be used to construct tests for diagnostic purposes. Bacteriophages also possess highly effective enzymes, which have had to adapt to their rapid development cycles. One of the main advantages is that, due to their compact genomes, it is very easy to modify them.

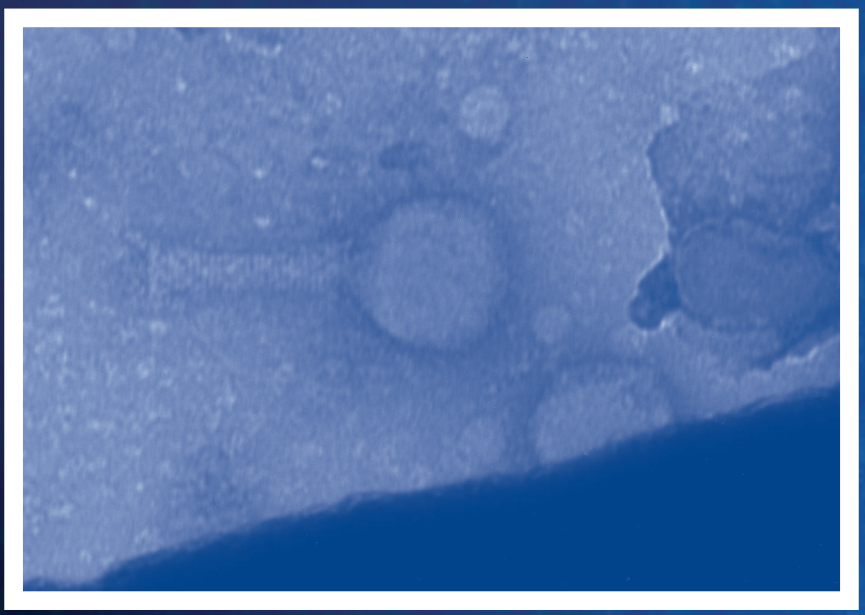

Figure 1: Bacteriophage T4 under electron microscopy

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Especially attractive is their ability to modify proteins that are exposed on the surface of capsid, which allows for use of the very robust technique of phage display. On the top of that, their production is relatively cheap.

Phage Display

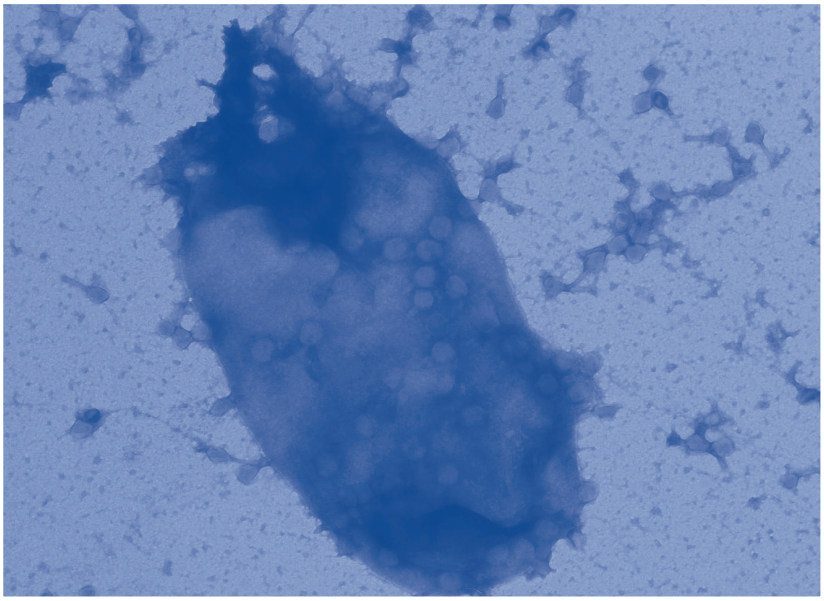
This technique allows for easy expression and selection of peptides that bind specifically to the selected target. Expressed peptides may be randomised or they may come from a cDNA library. This allows for easy access to cDNA libraries of antibodies expressed in animals such as camels or sharks. Following this, clones are selected which will bind to a given antigen and the selected antibody can then be easily sequenced and amplified in form of a phage, which expresses variable domains of antibody on the surface in the form of single chain fragment variable (scFv). When selecting scFv from randomised phage libraries – contrary to the use of libraries generated from lymphocytes of animals – it is also possible to obtain scFv, which binds to targets that are not immunogenic, such as metal oxides (4).

Another use of the phage involves the exact opposite – the ability to express almost any peptide on the surface of a phage makes it a very good carrier for the construction of vaccines. Moreover, their physical appearance reflects some mammalian viruses. Thus, when properly expressed, the peptides on the surface of a virus may look exactly the same as with the real virus.

DNA Vaccines

DNA vaccines are another example of the use of phage in biopharma. The concept relies on the ability of bacteriophage to deliver DNA to antigen presenting cells, which have a capability to express genes from cassettes inserted into phage genome. Such an approach leads to a slower build-up of immunity, but the final antibody titers may exceed those obtained by use of standard vaccines. Moreover, there are no obstacles when using this combined approach of a DNA phage vaccine with antigens presented on the capsid surface. This could potentially lead to rapid immunity build up with much higher antibodies titers present after complete immunisation (5).

Figure 2: Electron microghraph of bacterium killed by phage T4



Phage Therapy

Bacteriophages are a major concern in biopharma in cases where they constitute a major risk in bacterial fermentation (see Figure 2). However, this feature is beneficial if bacteria are to be killed. Phages as bactericide have some drawbacks, for example bacteria may become resistant to the phage. However, contrary to when using antibiotics, such resistance is seldom encoded on plasmids and thus is much harder for horizontal transfer. Also, phages can easily evolve countermeasures in order to bypass bacterial resistance to it (6). In this case, as both sides are biological entities which evolve and can mutate, it becomes very hard for bacteria to evolve resistance to the phage in a way which the phage cannot overcome. Even if such a situation did arise, it is easy to select alternate phages to which the bacteria would have not yet become resistant to. Moreover, phages have a better capacity for rapid evolution, as they multiply at much higher rates, offer a larger amount of progeny (up to several thousand progeny virions per phage with only two daughter cells per bacterium), and thus production of a mutation overcoming host defence is much more probable. However, if the naturally occurring high rates of evolution among phages are not enough, it is also possible to produce phages, which have pre-made variants of receptors and can bind to mutated versions of the host receptor (7). Such pre-adapted phages can quickly overcome mutations in host receptors, which otherwise would block phage adsorption and make bacteria immune.

The use of phage in therapy has been in use longer than sulfonamides. Their discovery was a great, but unfulfilled, promise to be a universal medicine against bacterial infections. After initial successful treatments, they later failed when used and prepared by unskilled people, who did not understand their nature, and thus their possibilities and limitations. Rapid commercialisation of the phage therapy was unreliable, and thus, when chemotherapy of bacterial infection became available, the phage therapy was discontinued. There was an exception from this rule – in Georgia phage therapy was administered and improved by professionals. Its commercialisation was not possible there, as it was a part of the Soviet Union at the time. This caused a particular

situation, whereby phage therapy was investigated using scientific methods, without the rush to create a profit out of it. For about 60 years, Georgia became the last bastion of phage therapy. Moreover, it appeared to be very successful there. The results obtained in Georgia, although very encouraging, lacked a double-blind test regime, and thus they could not be used for registration of bacteriophage cocktails as a therapeutic agent in most countries. This drawback was soon noticed, and new phage therapeutics were put into standard clinical trials. Their effectiveness was proven under this regime; for example, phage mix appeared to be effective in the treatment of chronic otitis caused by *Pseudomonas aeruginosa* in humans (8).

What is interesting is that phages do not have to be considered as a replacement for antibiotics. They act synergistically with some antibiotic classes. The result is the increase of phage burst size from the cell treated with some antibiotics. This allows phages to kill bacteria faster, as well as increases the effectiveness of antibiotics (9).

Phage Enzymes

Most bacteriophages in their life cycle use various enzymes in order to enter the bacterial cell, and then destroy it to release phage progeny. These enzymes are extremely active, and thus they may be used for rapid bacteria inactivation. Moreover, these enzymes utilise structures in bacterial cells, which are difficult, or even impossible, for bacterial cells to modify. The reason for this is the very long co-evolution of phages and bacteria, where selected phages target such structures. This field of research seems to be very promising, as enzyme action is almost instant (10).

Bacterial Detection and Phage Antibodies

Other bacterial proteins may soon be used in bacteria detection assays. Phage receptors have proven to be very stable proteins, showing a much higher resistance against unfavourable environmental conditions than antibodies. They have good potential for replacing antibodies in standard bacteria-detection assays and for this purpose it is possible to use whole phage particles. This approach mimics phage typing to some extent, which relies on the ability of various phages to multiply on given bacterial strains. The difference is that phages are just used to capture bacteria and generate a detectable signal (11). Although the ability of native phages to bind specifically is limited to bacteria, it is possible to generate and use it in the detection assay phage antibodies. This is created using the aforementioned phage display technique.

Except for phage typing – a well established method for strain identification – there are other types of phage-based detections which utilise the ability to multiply in host cells. Phage multiplication can be an indicator of the presence of pathogenic bacterial cells in samples (12). There are a few approaches; for example the detection of phage multiplication by plaque assay or by the incorporation of the luciferase gene into bacteriophage genome, which makes bacteria glow upon phage infection (13,14).

Phage Production

To use phages in any of the previously mentioned methods, it is necessary to first of all produce it. Phage production is relatively cheap, as it is based primarily on high density bacterial fermentation. It tends to be very effective, as icosahedral phages may obtain titers of 10^{13}/ml (15). This offers excellent possibilities, as 10^{8} phages is enough for the construction of sensitive ELISA-like assays for the detection of bacterial cells (10). However, phage production is challenging for a number of reasons. Originating from the process itself, there is the need for optimisation in order to obtain high production yields. A more problematic aspect can be the behaviour of infected culture, especially when cultures are infected by lytic phages that tend to foam extensively and may cause problems in the suppression of foaming, causing damage or clogging up the exhaust filter. Some phages are extensively resistant to harsh conditions, and there is the risk of problems in equipment cleaning and sterilisation. Another problem is contamination, which easily occurs in such facilities during material handling, potentially affecting facility productivity. Damage can range from premature infection in bacterial culture, which leads to sub-optimal productivity and lower phage yields, through the mixed phage infections, where a contaminating phage develops in culture in parallel with the produced phage. Released phage may also cause generalised facility contamination, leading to total paralysis of the facility.

The problems with phage production increases dramatically during scale-up processes. It is much harder to control foaming in large fermentation volumes, in the same way that it is harder to

properly clean and sterilise equipment. Also, the contamination of the facility is more probable when high volume fermentation is used. However, there are methods that allow for the production of relatively high volumes of phage lysate when employing relatively low volumes of fermentation. A method published by Sauvageau and Cooper employs a two-stage fermentation process, where the first stage delivers uninfected high density culture to the second fermentor where infection and phage propagation occurs (16). This kind of process can be run automatically, greatly improving the effectiveness of phage production in relatively small volumes of fermentation.

Conclusion

Phages are gaining more attention in biopharma, as well as in related fields, as they emerge to be a versatile and effective tool. There is the hope that increased successful use of phages for our benefit will arise from our greater understanding of their mechanisms, structure and role in the environment. They seem to be an attractive option for the construction of a broad range of products relevant for human and animal health, and thus, they are more frequently present in R&D programmes. However, due to a relative lack of interest in phage biology in the past – as well as too few scientists skilled in various phage-related microbiological procedures – there has been, to some extent, a reluctance to employ phages in biopharma. Despite that, the great potential of phage-based technologies has already started the new age of phage.

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