FiberCell Systems
A BETTER WAY TO GROW CELLS

Using 3-D Cell Culture Systems to Overcome Barriers in Recombinant Protein Production

Once upon a time, purifying proteins for research or therapeutic purposes involved a laborious extraction process with large quantities of starting material.[1] All of that changed with the discovery and utilization of recombinant DNA. Suddenly, producing proteins seemed as simple as moving DNA into a host cell and waiting for endogenous transcription and translation mechanisms to take place.[1] Recombinant proteins revolutionized the life sciences, giving scientists a bevy of new research tools. It also revolutionized medicine, with researchers developing hundreds of biologics to treat diseases ranging from diabetes to cancer.[2] However, some proteins, both natural and synthetic, cannot be produced using standard cell culture and bioproduction methods. New 3-D cell culture systems such as hollow fiber bioreactors (HFBRs) can remove these roadblocks and open new possibilities for recombinant protein production.

Making Recombinant Proteins

To produce recombinant proteins, scientists introduce recombinant DNA containing genes of interest into host production cells, often with the aid of plasmid or viral vectors. Once exogenous genetic material is inside the cell, it is processed by the host's transcriptional and translational machinery, resulting in protein production. Ostensibly, recombinant protein production continues as long as the gene of interest remains inside the host cell, although in practice, many factors affect recombinant protein production yield and quality.[1]

Prokaryotic cells such as bacteria and yeast are popular for recombinant protein production for several reasons. First, their molecular biology and physiology is well characterized and documented, resulting in fewer unforeseen effects. Second, they multiply rapidly and are easy and inexpensive to maintain. Finally, prokaryotic expression machinery can be stimulated by strong inducible promoters, offering scientists a modicum of cellular-level control over production throughput. These factors make prokaryotic cells very popular for large-scale protein production.[3,4]

However, complications arise when using prokaryotic cells to produce eukaryotic proteins. Because the cellular machinery is less complex, prokaryotic cells cannot match eukaryotic cells, which commonly produce fully functional, properly folded multiunit proteins.[3] Prokaryotic cells struggle to produce multidomain proteins, and while renaturation and refolding can be done in the laboratory post-production, it is cumbersome and may not yield functional proteins.[3]

Moreover, bacteria cannot generate post-translational modifications (PTMs) because they lack endoplasmic reticula and Golgi apparati. This is the biggest problem facing prokaryotic recombinant protein production because PTMs, including glycosylation, phosphorylation, and acetylation, are critical for protein folding, processing, stability, and overall functionality.[3,4] As such, mammalian cells are often preferred in situations where consistency and functionality are critical, such as therapeutics manufacturing.

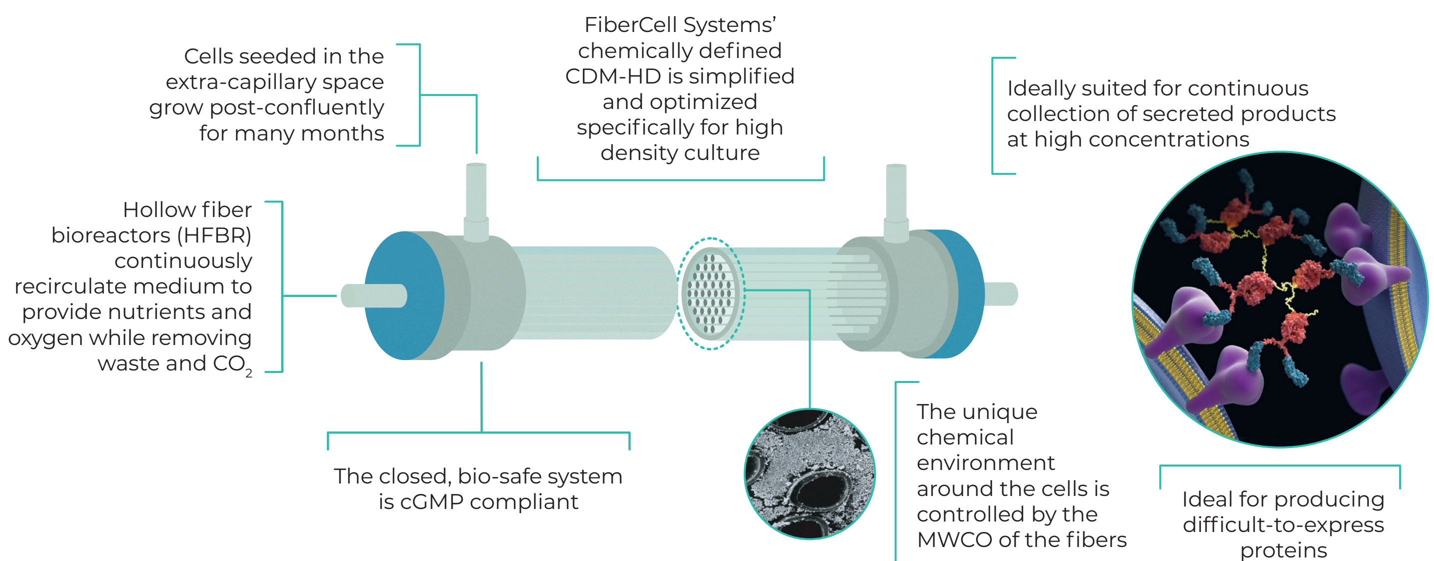
While mammalian cells produce physiologically-relevant proteins with more native structures and functionalities than bacteria and yeast counterparts, they also have their own problems. Culturing mammalian cells, for example, is more complex than culturing prokaryotic cells, as mammalian cells are more sensitive to changes in environmental conditions, ranging from shifts in pH to geometric arrangement. Mammalian cells respond to unfavorable environmental conditions by altering protein production mechanisms, resulting in dramatic shifts in yield and product quality.[3,5,6] Indeed, in the

absence of an in vivo-like environment, even mammalian cells can struggle to recapitulate key protein features such as dimerization and proper folding.

Potential Revealed: Introducing HFBRs

The need to recapitulate in vivo conditions for cell culture sparked interest in 3-D culture systems such as HFBRs. HFBRs place a multitude of ~200 μm-diameter porous hollow fiber filters inside a closed cylinder cartridge. This design increases the available surface area and capacity, with a medium-sized cartridge capable of supporting two billion cells. The fibers have a molecular weight cut-off (MWCO) limit of 20 kilodaltons (kDa) that controls what can cross from the media to the cells and vice versa. This MWCO value ensures that secreted products are trapped by the fibers, enabling high concentrations and easy harvesting.

Different Conditions Call for Different Media



The HFBR system also mimics the nutrient delivery and flow mechanics of the circulatory system, constantly supplying fresh nutrients to cells and removing metabolic byproducts. Rather than serum, which is not present under physiological conditions, cells inside HFBRs can be cultured using media containing CMD-HD, a chemically-defined serum replacement specifically designed for high-density cell cultures. In fact, the difference between high-density and low-density culture requirements is so great that CMD-HD will not work in flask or other low-density culture systems. CMD-HD does not contain proteins, steroid hormones, peptide mitogens, or animal-derived components. Instead, it uses recombinant growth factors, micro-nutrients, and customized ratios of amino acids and metabolites to better mimic in vivo high-density microenvironments. Together with CMD-HD, HFBRs offer better overall cell culture conditions, leading to more consistent and physiologically-relevant protein production, folding, and assembly that can continue uninterrupted for months at a time.

Using HFBRs to Increase Yield

Studying recombinant proteins can require large amounts of purified proteins. Traditional flask culture systems offer limited protein production abundance, requiring scaling-up. This is a major obstacle for researchers, particularly in the academic sector, who run smaller-scale culture setups due to logistical and economic obstacles. However, a single C2011 HFBR cartridge can support 1-2 billion cells, representing roughly the equivalent of 20-40 roller bottles or a two liter spinner flask.

This has opened new possibilities for researchers, especially for those examining the extracellular matrix. Investigations of extracellular matrix protein structures and their functions can necessitate

as much as milligrams of source material if researchers need to characterize a whole system.[7] Faced with this dilemma, scientists from the University of Manitoba in Winnipeg, Canada investigated if HFBRs could consistently generate high-quality recombinant Netrin-1 in greater abundance.[7]

Netrin-1 is an extracellular matrix proteoglycan that acts as an axon guidance cue, is difficult to produce because of highly variable expression and varied glycosylation patterns. Starting out in flask culture, this team increased protein yield from 1 mg/L to 15 mg/L using optimization techniques. However, their production remained inconsistent. They thus turned to HFBRs for their ability to mimic in vivo cellular growth conditions and support greater cell densities. The researchers identified a high-producing, stably-transfected human embryonic kidney (HEK) 293 clone and seeded HFBRs with it at an initial density of 10^7 cells. After experimenting to find the optimal doxycycline concentration for inducing protein production, they were able to reach consistent production at concentrations of ~175 mg/L, almost twelve-fold greater than what they had achieved with flask-based culture.[7]

The continuous flow present in HFBRs allowed these researchers to avoid low nutrient levels and decrease waste product recirculation during protein production, avoiding issues caused by the accumulation of byproducts such as lactate and ammonia. They were able to obtain a constant protein yield at each collection timepoint over a five-month duration. Moreover, collections entailed processing only 20 mL volumes, rather than >500 mL when using flask culture methods, with no decrease in quantity, thus improving throughput.[7]

High protein production can strain cell metabolism, potentially impacting protein quality. As such, the research team carefully examined the properties of their Netrin-1 end product. No precipitation was observed during purification, and SDS-PAGE revealed minimal molecular weight heterogeneity. Size-exclusion chromatography (SEC) and particle size distribution analysis both showed a single peak, indicating minimal aggregation. The researchers then investigated the behavior of the recombinant Netrin-1 in solution using SEC-MALS, which again confirmed minimal aggregation while showing the expected presence of monomers and dimers in equilibrium. Critically, these parameters evaluating protein quality did not change over the five-month production period.[7]

Overall, the Manitoba team were able to increase yields dramatically, improve production consistency, and achieve high product quality. This shows that HFBRs are a powerful tool for producing large amounts of protein for ECM research.

Overcoming Production Obstacles with HFBRs

Poor or non-physiological cell culture conditions negatively impact recombinant protein production, leading to inconsistent product yield and quality. By optimizing culture conditions, HFBRs can facilitate the recapitulation of physiologically-relevant protein assembly, folding, and post-translational modifications. This is vital to producing “difficult-to-produce” proteins, which drive many key physiological and pathological mechanisms.

For example, researchers from the U.S. National Cancer Institute recently used HFBRs to produce naturally-processed, biologically-active heterodimeric IL-15/IL-15Rα complexes.[8] Because IL-15 functions as part of a complex with IL-15 receptor α (IL-15Rα) that is formed intracellularly in the endoplasmic reticulum, both IL-15 and IL-15Rα need to be simultaneously expressed in the same cell to produce functional recombinant IL-15.

Producing two recombinant proteins in the same cell simultaneously is highly complex. However, large quantities of the IL-15/IL-15Rα heterodimer cannot be isolated from the circulation, and efforts

to produce the two monomers separately and then induce dimerization have been largely unsuccessful. As such, producing the recombinant IL-15/IL-15Rα heterodimer structure at once is necessary in order to generate sufficient yields for research and potential clinical applications.[8]

Taking advantage of the HFBR's ability to better recapitulate in vivo environments, Pavlakis' team stably transfected HEK293 cells and cultured them in HFBRs for five months. This workflow produced soluble IL-15/IL-15Rα heterodimers at yields of up to 70 mg/L for the entirety of the production span. With a reliable and ample supply of the IL-15/IL-15Rα heterodimer, the researchers were subsequently able to thoroughly characterize the properties of the complex, identifying the amino acid sequence, multiple glycosylation sites, and the proteolytic cleavage site of IL-15Rα. They were also able to examine the in vivo bioactivity and pharmacokinetics of the heterodimer, showing sustained elevation of plasma IL-15 levels as well as robust NK and T cell expansion in mice following administration. This characterization work helped establish IL-15 as a strong candidate for further evaluation for clinical use.[8]

In addition to facilitating in vivo complexing during protein production, HFBRs also aid in the generation of synthetic proteins that are challenging to manufacture with traditional flask culture methods. A research team from the Uniformed Services University of the Health Sciences demonstrated this by pioneering a novel method using HFBRs to produce uniformly trimeric recombinant human cytomegalovirus (HCMV) glycoprotein B (gB) protein.[9]

Researchers are interested in studying HCMV gB as a major target antigen to create an HCMV vaccine. The wild-type HCMV gB protein has a furin cleavage site, which is necessary to fold the protein into its natural trimeric form. However, the furin cleavage site also impedes recombinant HCMV gB production. When researchers removed the site, they obtained high yields, but generated only monomeric products. The Uniformed Services University of the Health Sciences team replaced the furin cleavage site with a 15-amino acid linker, hoping to avoid yield issues while still maintaining trimer formation. They transfected Chinese hamster ovary (CHO) cells with the coding sequence for this altered HCMV gB and cultured the CHO cells using HFBRs. Western blot analysis of the resulting recombinant protein product showed a single ~360 kDa band indicative of trimer formation (the HCMV gB monomer is roughly ~120 kDa in molecular weight). Additionally, SEC-MALS revealed a single 728 kDa peak, consistent with a dimer formed from two HCMV gB trimers.[9]

The ability to produce recombinant trimeric HCMV gB allowed researchers to examine how trimeric gB performed relative to monomeric gB in terms of eliciting antibody production. Administration of the recombinant trimeric protein to mice induced up to 11-fold higher serum titers of total gB-specific immunoglobulin G (IgG) compared to monomeric HCMV gB. Furthermore, immunizing mice with trimeric HCMV gB resulted in higher neutralizing activity for fibroblasts and epithelial cells. These data strongly indicate that trimeric HCMV gB should be clinically evaluated as a prophylactic HCMV vaccine candidate.[9]

Opening Doors

Recombinant proteins are a great asset for research and therapeutics, but fully recapitulating in vivo protein structures and functions remains a challenge. 3-D cell culture systems such as HFBRs give scientists a better way to replicate in vivo environmental conditions, thereby paving the way for more physiologically-relevant protein production. HFBRs also offer considerable advantages in terms of maintaining consistent production over prolonged periods of time, as well as achieving higher product purity and yield. Researchers, particularly in the academic sphere, are taking advantage

of these systems to overcome supply and logistical issues to better characterize protein structures, generate experimental materials, and identify new therapeutic possibilities.

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