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Environmental considerations in biologics manufacturing

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Received 4th January 2010, Accepted 19th February 2010
First published as an Advance Article on the web 12th April 2010
DOI: 10.1039/b927443j

This perspective originated from our initial environmental assessment of biologics manufacturing as an extension of earlier work on small-molecule pharmaceutics spearheaded by the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR). Systematic analysis was focused on therapeutic proteins due to their current predominance in biotherapeutics. The E factor for process water was found to represent an important environmental index primarily because aqueous solutions are used in practically every processing step, and significant process improvements typically result in sizable reduction in the usage of water and associated chemicals. Compared to small-molecule drugs, manufacture of therapeutic proteins by fermentation requires approximately 10 to 100 times more water per kg of product, but very small amounts of solvent, especially hazardous ones. The amounts of solid waste generated from consumables are comparable between the two groups. A great deal of water is also consumed for non-process operations at bioprocessing plants, which necessitates an E factor for non-process water to help monitor this part of plant operation. Useful environmental indices for biologics manufacturing should also include energy consumption, reportedly dominated by facility operations, especially for cleanroom or controlled space because of the required HVAC (Heating, Ventilation, and Air Conditioning) for its operation. Notable emerging developments for therapeutic protein production include biogenerics, novel bioprocessing technologies, process analytical technology (PAT), single-use (disposable) manufacturing, and alternative production platforms such as cell-free synthesis and transgenic plants or animals. The potential impact of these technologies from an environmental standpoint is discussed.

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1. Introduction

The pharmaceutical industry is making a conscientious effort to develop cleaner and more efficient processes for manufacturing small-molecule drugs. This development has been guided by the principles of green chemistry and engineering that stress

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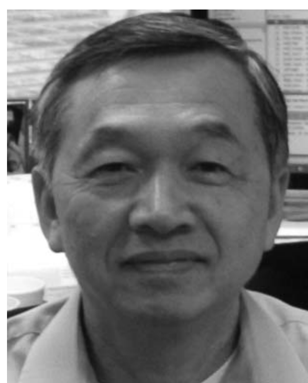

Sa Ho

Sa Ho is a Senior Research Fellow in Pfizer Biotherapeutics Pharmaceutical Sciences where he has been involved in developing new technologies and processes for biotherapeutics manufacture since 2002. After receiving a PhD in Chemical Engineering from Cornell University, he joined Merck, then moved to Monsanto, where he spent almost 20 years in its Corporate Research department. His industrial R&D career spans small molecules, biologics and environmental fields. In addition to successfully developing many bioprocesses and membrane separation technologies, at Monsanto he led a large industry–government consortium in the development and implementation of an innovative, patented technology, Lasagna®, for in situ *remediation of contaminated soils.*

Joseph McLaughlin

Joseph McLaughlin is an Associate Research Fellow in Pfizer Biotherapeutics Pharmaceutical Sciences with 25 years experience in biotechnology process development and technology transfer. He began learning about biotechnology at Rice University, where he earned a Master of Science in 1983 to complement his BS in Chemical Engineering from the University of Texas. Joseph has worked in both upstream and downstream process areas on a wide range of microbial and mammalian sourced products. Joseph is now a member of Pfizer's Bioprocess Research and Development Manufacturing Group responsible for pilot scale process development and preparation of Drug Substances for clinical trial supplies.

prevention such as atom economy, less hazardous chemical synthesis, use of safer chemicals, design for energy efficiency, and use of renewable feedstocks.[1,2] The work has been spearheaded by the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR), a coalition between the ACS Green Chemistry Institute (ACS GCI) and a number of major pharmaceutical corporations or companies with a strong interest in Pharmaceutical Manufacture (Merck, Pfizer, Eli Lilly, AstraZeneca, Schering-Plough (now part of Merck), GlaxoSmithKline, Wyeth (now part of Pfizer), Boehringer Ingelheim, Johnson and Johnson, Novartis, Roche, Abbott, DSM, Dr Reddy's and Codexis) with the charter of integrating the principles of green chemistry and engineering into the business of drug discovery, drug development and production.

The ACS GCIPR group applied the process mass intensity or E factor concept originally developed by Sheldon for the chemical industry[3] in analyzing the overall greenness of pharmaceuticals production. E factor is defined as the total amount in kilograms of organic solvents, reagents, and consumables used per kilogram of product produced. Reviewing 19 development projects from the company members, the group reported water usage on the average of 50 kg per kg product with a range of 10 to 250, and solvents of 100 kg per kg product with a range of 20 to 440, 90% of which are considered hazardous or flammable solvents.[4,5]

With the advent of molecular biology and supported by enhanced large-scale bioprocessing capabilities, biotherapeutics – biological compounds used for treating diseases – have emerged in the last two decades as an important class of drugs and are now an integral part of product portfolios in most if not all major pharmaceutical firms. Biotherapeutics complement small-molecule drugs by expanding accessible targets and, for many indications, provide uniquely effective therapies. A particular group of proteins called monoclonal antibodies has been extensively employed and holds great promise as therapeutic agents for their highly specific binding to cellular receptors as well as for their integral roles in the body's immune system. Clinically, therapeutic proteins have contributed essential therapies to critical diseases, many life-threatening, including diabetes (insulin), end-stage renal disease (erythropoietin), viral hepatitis (interferon or IFN), cancer (trastuzumab for metastatic breast cancer, bevacizumab for metastatic colorectal cancer, I-131 ch-TNT for advanced lung cancer), growth anomaly (human growth hormone and its antagonist), clotting disorders (Factor VII, VIII, IX), rheumatoid arthritis (anakinra), multiple sclerosis (IFN-β1a and 1b), and inborn errors of metabolism (lysosomal enzymes).[6-8] Therapeutic vaccines represent an emerging area in which biologics are used to treat infectious diseases, autoimmune diseases and cancer, with Gardasil® as an example of a recently approved cervical cancer vaccine.[9]

It is thus of interest to extend the work of the ACS GCIPR group to biotherapeutics. Most biologics, especially proteins, are produced by fermentation, not chemical synthesis. Biological processes are generally considered natural and therefore inherently green. Therapeutic biologics, however, span a very broad range of compounds (peptides, proteins, antibodies, nucleotides, and many forms of vaccines) with highly diverse properties and correspondingly varied manufacturing processes. Systematic environmental assessment of these systems would first require grouping them into proper classes with common characteristics from a manufacturing standpoint. The perspective described in this chapter is drawn from an earlier ACS presentation[10] and represents our initial attempt to establish a general framework for consideration, which hopefully would encourage others in the biopharmaceutical industry to join in the effort.

2. Characteristics of therapeutic biologics

Highly diverse in properties and manufacturing processes, therapeutic biologics can be loosely categorized into four main groups as follows, with an eye towards implementing the E factor concept.

Berkeley Cue

Berkeley W. Cue consults with pharmaceutical and technology companies to create innovative solutions for pharmaceutical science and manufacturing challenges. At Pfizer he was responsible for Pharmaceutical Sciences at their Groton R&D site. He was a member of the Worldwide Pharmaceutical Sciences Executive Team, and sponsored the global Chemical R&D line council. He created and led Pfizer's Green Chemistry initiative and has spoken extensively on this topic since 2000. Dr. Cue retired from Pfizer in April 2004 after almost 29 years, but he continues his mission of advancing green chemistry in the pharmaceutical industry. Since 2004 he has given more than one hundred presentations on green chemistry in the pharmaceutical industry.

Peter Dunn

Peter Dunn completed his PhD at Imperial College, London, in 1987 with Professor Charles Rees. He undertook post-doctoral research at the ETH, Zurich, with Professor Albert Eschenmoser and at the University of California Berkeley with Professor Henry Rapoport. From 1989–2000 he worked in Chemical R&D at Pfizer as a project scientist and became the inventor of the commercial processes to make four medicines including Viagra™ and Emselex™. Between 2000 and 2006 he was Director of Chemical Research and Development in the UK. In 2006 he took up his current role as the Global Green Chemistry Leader for Pfizer.

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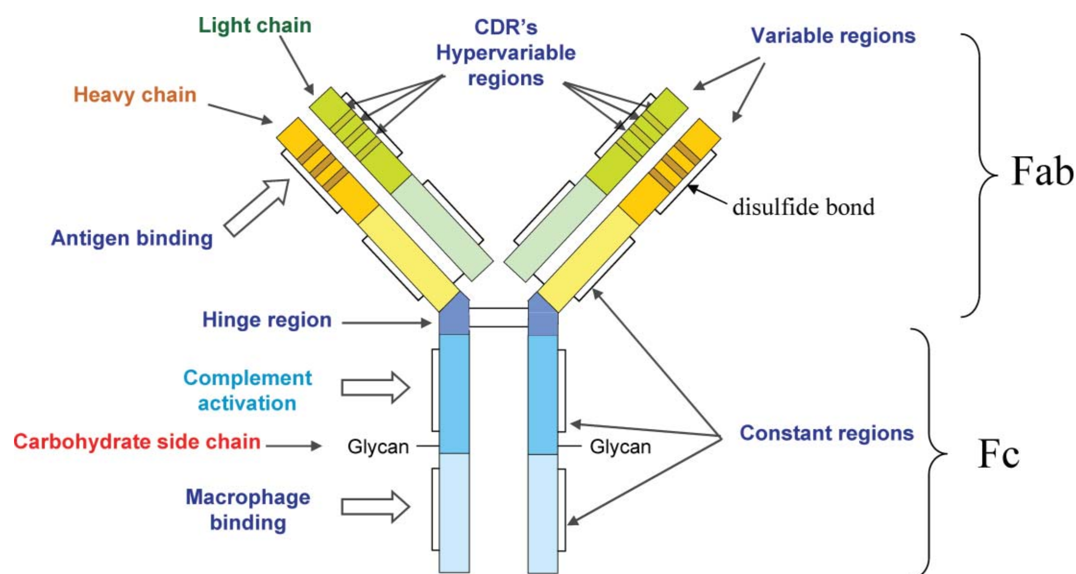

Fig. 1 General structure of IgG monoclonal antibodies (shown in the diagram is IgG1, a subclass of IgG, exhibiting two disulfide bonds between the two arms of the full antibody). (Reproduced with permission from Wiley-VCH).

2.1 Peptides

These are made up of approximately 20 to 40 amino acids, with molecular weights typically below 5000 Da, and produced by chemical synthesis, primarily *via* solid-phase synthesis. The manufacture of peptides is thus closer to that of small molecules than to fermentation-based processes.

2.2 Proteins

These are larger than peptides with molecular weights ranging from around 10 kDa to 200 kDa or higher, and are produced by fermentation using primarily microbes or mammalian cells. Proteins can be further subdivided into two main groups: monoclonal antibody and non-antibody proteins.

– Monoclonal antibodies (mAbs) designate a general class of compounds with defined structure and typically with molecular weight around 150 kDa, and are produced by cell culture using mammalian cells. Fig. 1 shows the general structure for IgG (immunoglobulin G), a common class of therapeutic monoclonal antibodies. An IgG molecule comprises a disulfide-linked pair of identical heavy chains, each intertwined with, and also disulfide-linked, to a light chain. The chemical (amino acid sequence) change from one mAb to another is primarily in the variable regions that contain short amino acid sequences call CDRs (complementarity-determining regions) where impurities, toxins or foreign substances (called antigens) are bound with high specificity. MAbs may have sugar groups attached at the position shown in the Fc region, a process called glycosylation, which can provide important biologic functions and increase the heterogeneity of the product. Fig. 2 shows a computer-modeled 3-D structure of human IgG2, a subclass of IgG, illustrating the complexity of these molecules.

– Non-antibody proteins represent a very large and diverse group with highly variable properties depending upon their original sources and biological functions. Recombinant proteins produced in microbes – mostly *E. coli*, some with yeasts – can vary widely in size, charge, hydrophobicity, and conformation. Additionally, bioactivity may require the target protein to be in its multimeric forms, that is, dimer or larger. The variation in size, for instance, ranges from 5.6 kDa for insulin, 22 kDa for human growth hormone to several million for a virus-like particle, which is actually a great assembly of over 100 monomeric protein units. Other emerging therapeutics that could involve proteins as part of the drugs include conjugated proteins, such as those attached to a polyethylene glycol (PEG) molecule, and therapeutic vaccines in which protein antigens are used to generate immune responses from the body against certain diseases.

2.3 Nucleotides

These are polymers of nucleic acids that genes are made up of and that are used as therapeutics for their binding properties or genetic functions. They can be divided into two groups:

– Oligonucleotides: these typically range from 20 to 40 nucleotides in length and are produced by chemical (primarily solid-phase) synthesis. An example of a commercial product is Macugen®, a 28-mer oligonucleotide covalently linked to a large polyethylene glycol (PEG) molecule. Like peptides, the manufacture of oligonucleotides is closer to that of small-molecule drugs.

– Plasmid DNA: these are circular strands of DNA, much larger than oligonucleotides (from 2 to over 10 kilobases, with molecular weight from a few hundred thousand to several million). They are produced by fermentation using microbial cells.

2.4 Vaccines

This group represents an entire class by itself with many different forms and functions. They span from the traditional vaccines such as inactivated or attenuated microbes or viruses to peptides and proteins, to DNA, to virus-like particles, and usually are in

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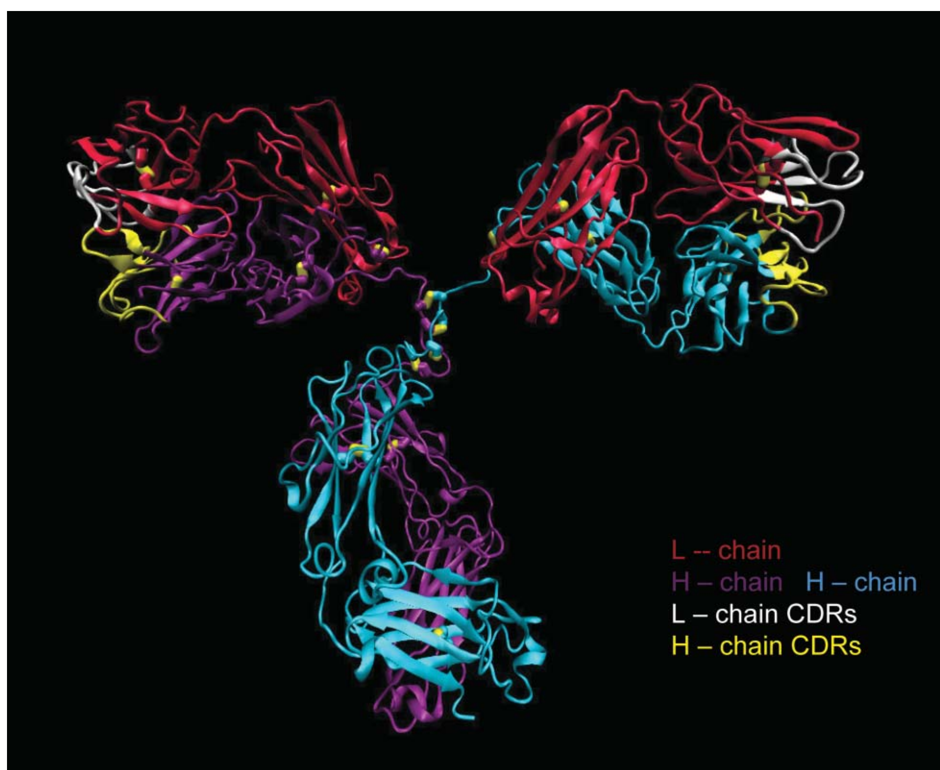

Fig. 2 A computer-modeled 3-D picture of IgG2, one of the IgG subclasses which differ in their disulfide bonding (yellow) patterns especially in the hinge region of antibody. (Generated by Xiaoling Wang and Sandeep Kumar).

combination with an adjuvant for enhanced efficacy. They are typically produced by fermentation, except for peptides, which are produced by chemical synthesis as mentioned above.

According to a review by Walsh,[8] of 165 biopharmaceutical products approved in the U.S. and Europe by 2006 only two are nucleic acid-based drugs, whereas nine of the 31 therapeutic proteins approved since 2003 are produced in *E. coli*, and 17 are produced by mammalian cell lines. In 2004 market distribution and manufacturing of therapeutic proteins, non-glycosylated (non-antibody) proteins constitutes 40% of the total market with 12% annual growth rate and are produced in *E. coli* or the yeast *Saccharomyces cerevisiae*; glycoproteins (primarily mAbs) constitute 60% of total market with 26% annual growth rate and are produced by mammalian cell culture (mostly with cells from Chinese Hamster Ovary, or CHO).

Not only do therapeutic proteins dominate the world of pharmaceutical biologics, they are also produced by biological processes as opposed to chemical synthesis, which is the method of choice for production of peptides and oligonucleotides. It thus seems appropriate to focus the green technology assessment on therapeutic proteins in order to complement the work of the ACS GCIPR on small-molecule drugs.

3. General features of therapeutic protein manufacturing

Manufacturing processes for therapeutic proteins can vary greatly, especially for non-antibody proteins. They, however, share some common features that differ significantly from those of small molecules. The general scheme for protein manufacturing is shown in Table 1. It typically involves a product synthesis step (bacterial fermentation or mammalian cell culture) followed with a series of processing steps, commonly called downstream processing (DSP), to recover and purify the protein of interest. A major difference with the manufacture of small molecules is the need to purify the target protein from a large number of different impurities present in the post-fermentation solution, which, in addition to the chemical reagents used in the process, include host cell components (proteins, DNA), and even various altered forms of the protein product itself. This diverse mixture of impurities is the main reason for the complexity of downstream processing that typifies the production of therapeutic proteins.

Raw materials and processing reagents commonly used in the manufacture of therapeutic proteins are shown in Table 2. This is another area where the manufacture of biologics differs greatly from that of small molecules. Owing to the complex machinery of biological cells, raw materials required for protein synthesis comprise mainly water, sugar, salts, trace minerals and some supplements. Similarly, the processing area uses mostly water and salts in buffer solutions, and consumables such as filters and chromatography resins. Very little organic solvent is used, if at all, and they tend to be non-hazardous such as alcohols.

One distinguishing characteristic of protein manufacturing is the extensive use of water, primarily a consequence of fermentation-based production and current purification practice. In the fermenter or bioreactor, concentrations of the protein product formed, called titers, are typically from 1 to 5 g per L. Yet, even a very high titer of 10 g per L is equivalent to only 1 wt% of the solution, which means that roughly 100 kg of water is already required per kg of

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Table 1 General process scheme for therapeutic protein manufacturing. (Reproduced with permission from Wiley-VCH)

Product synthesis
• Fermentation with bacteria (*e.g. E. coli*), yeasts or fungi for non-antibody proteins
• Mammalian cell culture for production of antibodies

Downstream processing
• **Isolation/recovery**
– *Product in fermentation broth*: cells and solid removal, volume reduction
– *Product inside cells*:
> Soluble form: cell disruption, solids removal, volume reduction
> Insoluble form (inclusion bodies): homogenization, differential centrifugation, wash, dissolution
• **Purification/reaction**
– *Bulk and intermediate purification*: primarily for removal of process-related impurities, *e.g.* reagents, host cell proteins, DNA, endotoxins; some product-related impurities; common methods:
> Precipitation, adsorption, extraction
> Chromatography (bind/elution, flowthrough)
– *Ultrafiltration/diafiltration* (*UF/DF*): used as needed for product concentration (volume reduction) and buffer exchange (prepared for next step or for storage)
– *Reaction*: used at an appropriate point in purification train for conversion to bioactive forms (*e.g.* refold/oxidation, dimer formation, PEGylation)
– *Polishing*: final purification step (invariably using chromatography) to remove close product-related impurities, residual host cell proteins (HCPs) and endotoxins.
– *Final UF/DF and sterile filtration*: concentration and buffer exchange for long-term product storage or preparation for drug product formulation

Table 2 Typical raw and processing materials used in manufacture of therapeutic proteins. (Reproduced with permission from Wiley-VCH)

Fermentation: Product synthesis
• Water, inorganic salts, caustic and acids for pH adjustment and cleaning
• Carbon source (glucose), nitrogen source, complex protein source (yeast extract, serum…), small amounts of organics, antifoam
Downstream processing: Purification/reaction
• Processing materials:
– Water
– Inorganic salts, bases and acids: pH adjustment, chromatography column operations, cleaning, and buffer solutions for storage
– Urea, detergents: to enhance solubilization or minimize aggregation of certain proteins
– C_2–C_5 alcohols and/or glycols for certain chromatographic modalities (hydrophobic interactions, reversed phase)
– Special organic solvents (*e.g.* CH_3CN): for post-fermentation modification or conjugation reactions such as PEGylation
• Consumables:
– Dead-end filters; disposable bags, tubing and connectors
– Ultrafiltration/microfiltration membranes
– Chromatographic resins

Table 3 Water usage for two common purification unit operations. (Reproduced with permission from Wiley-VCH)

	Typical operating range			
Chromatography column[a]				
Resin loading, g protein per L resin	10	20	50	100
kg Water per kg product	1000	500	200	100
Ultrafiltration/diafiltration[b]				
Protein concentration, g per L	10	20	50	100
kg Water per kg product	1000	500	200	100

[a] Chromatography column: number of column volumes (CVs) of buffer solution used is assumed to be 10; typical range is 10 to 20 CVs. [b] Ultrafiltration/diafiltration: number of turn-over volumes (TOVs) of buffer solution used is assumed to be 10; typical range is 5 to 20 TOVs.

unprocessed protein in the fermentation broth. Two commonly used unit operations in bioprocessing, column chromatography and ultrafiltration/diafiltration, also happen to consume large amounts of water. Shown in Table 3, typical water usage for these two units can range from 100 to 1000 kg water per kg product for each step. Actual water usage on the basis of the purified protein weight would be even higher because of product loss occurring in these steps as well as in the rest of the process.

4. Therapeutic protein manufacturing process assessment

Many factors affect the process design for production of therapeutic proteins; they include protein type and size, production scale, and the type of host cells used. The environmental impact consideration will be focused on two major groups: non-antibody proteins produced by microbial cells and mAbs produced by mammalian cells.

4.1 Microbially-produced proteins

Recombinant proteins are produced by fermentation using primarily bacterial cells, mostly *E. coli*, some with yeasts. The manufacturing processes are highly variable in complexity, primarily because downstream processing has to adapt to the particular host expression system and the properties of the target protein itself.[11-15] They range from the very complex production process for insulin to a well-optimized process for a mature protein product in large-scale commercial production.

Human insulin is a small protein consisting of 51 amino acids with a molecular weight of 5734 and is made up of two peptide chains connected by two disulfide bonds. This small recombinant protein has been produced on very large scales for over two decades. The various commercial processes in

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production have been improved over the years but are still very complex.[16-19] The insulin manufacturing process discussed here is similar to the Eli Lilly's commercial process and is taken from a textbook on bioprocessing.[19] The process consists of a fermentation step to produce proinsulin and a highly complicated downstream processing train to recover proinsulin from the *E. coli* cells in the fermenter, convert it to insulin, then purify it to meet the required product quality for use in humans. For each kg of insulin produced an enormous amount of process water is consumed (>30 000 kg) along with over 4000 kg of organic solvents, some which are hazardous. Also about 15 kg of consumables (solid processing aids) are used per kg of insulin produced. These values, especially for water and solvents usage, are very high and represent a unique, extreme case for manufacturing of biologics due to the length of the purification process and the required complex reaction steps that are unusual in biologics processing.

A typical manufacturing process for a medium-sized protein produced by microbial cells would have a fermentation titer of 1 to 5 g per L and consist of 3 to 4 chromatographies with 2 to 3 UF/DF steps and up to 1 reaction step. The overall yield of this process from fermentation to API would range from 15 to 30% with no recycling or recovery of used materials. The water usage for this typical process is estimated to range from 10 000 to 20 000 kg of water for every kg of protein produced. The amounts of organic solvents could be substantial if certain purification steps such as reversed phase chromatography are used. Some hazardous solvents may be used to carry out chemical reactions such as conjugating another molecule, such as PEG, to the protein to enhance its specificity and/or stability. For consumables, the amount ranges from 10 to 30 kg per kg protein product. Glucose is the main raw material for cell growth and urea is commonly used for solubilizing inclusion bodies and in assisting the conversion of a protein to its active conformation.

Greener approaches in manufacturing would strive toward a minimum number of downstream processing steps, high overall yield, and recycling water and key processing materials. The commercial production of bovine somatotropin (BST), a growth hormone for increased milk production in dairy cows, represents a real-life case in which these benefits were realized. In addition to high titers (5 to 10 g per L broth), the protein is produced as inclusion bodies in the cells, which facilitates their recovery with relatively high purity from the fermenter by simple homogenization and differential centrifugation. After dissolution of inclusion bodies followed with a simple refold step to form the bioactive BST, the purification consists primarily of a precipitation step to remove the bulk of the impurities and then a single chromatography column as a polishing step. As shown in Table 4, the amount of water used is reduced to less than 500 kg per kg of BST produced, and with very little urea consumption,[20] which is remarkable due mostly to the recycling of water and urea. While there is no cost driver for recycling of process water, the imposed environmental constraints at the manufacturing site on urea discharge necessitate its recycling as aqueous solutions, resulting in water itself being recycled as well. The consumables used are also quite low, about 4 kg per kg of BST. This real-life example demonstrates that a highly-optimized biologics production process with incorporated recycling of materials can drastically reduce both consumption of water and generation of chemical and solid wastes.

Table 4 Water/materials usage for the commercial Bovine Somatotropin (BST) manufacturing process. (Reproduced with permission from Wiley-VCH)

	kg per kg BST
Materials	
Glucose	96
Salts	8
(Reverse osmosis) water[a]	454
Urea[a]	26
Consumables	
Fermentation filters	3
Aseptic filters	1
Chromatographic resin	~0.1
UF membrane	~0.1
Total consumables	**~4**

[a] With urea and water recycling.

4.2 Monoclonal antibodies and mammalian cell culture processes

From the manufacturing standpoint, three main characteristics differentiate the production of mAbs by mammalian cells from production of non-antibody proteins by microbial cells. First, the fermentation process during which the protein product is formed is much longer for mammalian cells than for microbial cells, about 14 days *versus* 2 days with *E. coli*. Second, the protein produced by mammalian cells is generally secreted into the culture medium, negating the need for cell lysis to recover the product and thus avoiding release of host cell components into the culture solution. Finally, IgG antibodies bind selectively to protein A, enabling the use of a protein A affinity chromatography step to capture mAbs with high yield and purity from the clarified fermentation broth.

Many excellent reviews on monoclonal antibody manufacturing have been published.[21-24] Given the common properties of mAbs noted above, their manufacturing processes have become more and more standardized over the years with enhanced efficiency. Typically, after the fermentation step (called cell culture for mammalian cells), the cells are removed by centrifugation and depth filtration to obtain the clarified broth containing the product protein. The traditional "platform" purification process consists of 3 chromatography columns: a Protein A affinity column where the mAb product is concentrated and host cell proteins and genetic components (DNA) along with cell culture media are removed, an ion-exchange column as an intermediate purification to further remove host cell impurities and aggregate form of mAb, and finally another ion-exchange column (or hydrophobic interactions chromatography) as a polishing step to remove residual impurities. Viral clearance is a major issue with mammalian cell culture processes and is carried out with two different, orthogonal steps, as required by the FDA, typically a chemical virus inactivation step at low pH and a viral filtration step for physical removal of the viruses.

Manufacturing processes for mAbs range from older ones still in production to newer and more optimized processes; the

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Table 5 Order-of-magnitude estimate of process water and materials used in manufacture of therapeutic proteins (all values in kg per kg API). (Reproduced with permission from Wiley-VCH)

	Microbially-derived proteins		mAbs from cell culture	
	Highly optimized, large-scale	Typical "composite" process	Optimized large-scale	Highly intensified, large-scale
Water usage	<1000	15000	4500	1500
Salts + buffers	1	400	300	100
Consumables (solid wastes)	1	20	4	2
Organic solvents	~0	100 (alcohols, may involve some hazardous solvents)	8 (alcohols)	8 (alcohols)

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latter group typically consists of one to multiple bioreactors operating in parallel to deliver cell culture broth to a single purification train. The product formation step typically involves 1 to 6 bioreactors of up to 20,000 L each, with mAb titers from 2 to 5 g per L broth. Downstream processing includes a 3-column purification train with one of the last two columns being a flow-through. Annual throughput ranges from 400 to 5000 kg mAb, with an overall yield from cell culture to purified mAb of around 65 to 80%, and no recycling or recovery of process chemicals. Typical mAb production processes consume water from over 3000 to almost 7000 kg per kg mAb produced and consumables from 2 to 8 kg per kg of mAb. Water usage in the cell culture step makes up 20–25% of the total whereas the three chromatography columns use over 50% of the total. Furthermore, the large buffer amounts required as scale increases can result in WFI costs representing a surprisingly large proportion of the costs which can, in some cases, be greater than the cost of other raw materials.[23] Hence, it is expected that strategies that lower buffer, and hence WFI consumption, will potentially lead to significant environmental and financial benefits.

Strong advocacy has been made for process intensification to handle highly productive cell culture titers of 10 g per L or higher in a large-scale mAb manufacturing plant (10 ton of purified mAb per year) utilizing conventional unit operations.[25] Only two chromatography columns are used in the process and very high resin/membrane loadings are assumed. With this process intensification, the amount of water used drops to 1500 kg per kg mAb, which is about half of that for a typical mAb process. The total consumables used (chromatography resins, prefilters, viral filters and membranes), estimated from the data provided in the cited publication, are around 2 kg per kg mAb, with prefilters constituting 70% of the total weight due to their prevalent use in bioprocessing.

5. Overall environmental considerations

As noted, within each group (microbial and mammalian) less water is consumed as the process becomes more efficient, indicating that the E factor based on water usage would be a strong indicator of the degree of greenness in the production of therapeutic proteins. This reflects the fact that every step in the manufacturing process uses aqueous solutions, which in turn require chemicals (salts and buffers) and consumables (filters, resins, membranes, disposable bags) for processing. Process water and materials used in the manufacture of therapeutic proteins, both mAbs and non-antibody proteins, for the cases discussed above are summarized in Table 5. Table 6 contrasts water usage and solid waste generation for production of small-molecule drugs *versus* therapeutic proteins. If insulin is disregarded as an atypical case for biologics, small-molecule processes can be seen to require a great deal less water but significantly more solvents, especially hazardous ones. Solid waste generation (filter, resin, catalysts, etc.) seems comparable between the two systems.

Total water usage at biotherapeutics manufacturing plants, however, includes a great deal more operations than just direct process water to make products, such as in equipment cleaning, generation of water for injection, treatment of biowaste streams (~1 to 2 kg steam needed per kg waste solution), facility maintenance (cleaning, cooling/heating, etc.), and evaporative loss. Genentech published on its website[26] the average amount of water usage for all its manufacturing sites for the period of 2004 to 2006. The numbers reported are on the order of several hundred thousands kg water used per kg of protein produced. Approximate estimates for a Pfizer pilot plant and a small manufacturing facility appear to be in the same order of magnitude. These large numbers for water usage at a biotherapeutics manufacturing plant highlight the tremendous opportunity

Table 6 Comparison of water and materials usage between small-molecule drugs and therapeutic proteins (all values in kg per kg product). (Reproduced with permission from Wiley-VCH)

	Small molecules	Therapeutic proteins		
	19 Developmental compounds	Insulin	Medium-sized proteins	Monoclonal antibodies
Process water[a]	50 (range: 10–250)	34000	1000–20000	1500–4500
Organic solvents[a]	100 (range: 20–440)	1600	0–200 (primarily alcohols)	~10 (primarily alcohols)
Hazardous or flammable solvents	>90% of total organic solvents	500	0–5	None
Consumables (solid wastes)	<5	14	1–30	2–4

[a] From Pharmaceutical Roundtable bench-marking results.

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for reducing water consumption, and associated chemicals, through better plant design, more streamlined operations, and particularly water recycling.

In addition to water and consumables, energy is used in the production of biotherapeutics not only to operate the process but also to maintain a controlled environment, to produce clean utilities, and to clean and sanitize equipment. Frequently the major energy cost considered in economic analysis is the HVAC (Heating, Ventilation and Air Conditioning) cost. These controlled environments must be validated and maintained in continuous operation to assure that the product is not contaminated by the environment. Thus different levels of environmental control and their associated HVAC requirement strongly affect the overall energy usage at a biotherapeutic manufacturing plant, and hence cost.[27] A benchmarking study of cleanroom energy use in high-tech and biotech industries for three representative facilities shows that HVAC energy use ranges from almost 40% to over 60% of the total energy, whereas energy for process and process utilities ranges from about 50% to less than 20% of the total.[28] More efficient design and operation of the controlled environment represents an important opportunity in moving toward a "greener" facility.

Clean utilities such as WFI can be designed for very energy-efficient operation by using approaches such as vapor re-compression for water distillation. Equipment cleaning and sanitization consume a significant amount of energy because they typically require high flow rates and large temperature changes. A typical biotherapeutic process uses some energy in the upstream operations for heat and mass transfer (agitation and sparging) and only a modest amount of energy for fluid transport and mixing in downstream operations. Overall, an improved understanding of the use of energy in biotherapeutics manufacturing as well as innovative development to *minimize* the requirement of cleanroom space itself could significantly reduce the environmental impact of facilities, especially at the earliest stages of architectural design.

For biologics manufacturing in general, from an environmental sustainability standpoint, it is worth considering the two main variables that have been proposed in other industries to provide a balanced view of the environmental impact of inputs (resource usage) and outputs (emissions, effluents and wastes), and the products and services produced.[29] They are termed Natural Resources Sustainability (NRS) and Environmental Burden Sustainability (EBS). NRS is based on evaluation of 4 main variables: energy, materials (excluding fuel and water), water, and land. These variables are related to economical considerations depending upon the different markets. EBS addresses pollutants as they are released to the atmosphere (emissions), water (effluents), and soil (solid wastes). Each substance is tied to a weighting factor known as the "potency factor" that draws on developments in environmental science to estimate potential environmental impact rather than based solely on the quantities of the materials discharged.

6. New developments with potential environmental impact

Emerging areas with potential environmental impact are driven by business objectives as well as advances in the technical arena. Listed in Table 7, these span quite a diverse range of developments that includes biogenerics, process and analytical technologies, single-use (disposable) manufacturing, and alternative production platforms such as transgenic plants or animals. The environmental implication of each group is briefly discussed here. Among them, single-use manufacturing will likely have a large impact on protein production in the near term, and some alternative production platforms, if successful, could potentially alter the landscape of protein manufacture in the long term. While comprehensive environmental assessment of these two areas is beyond the scope of this article, a focused review of their significance is warranted.

Table 7 New developments with potential environmental impact

- Biogenerics
- Process and analytical technologies
  - Cell line and bioreactor optimization: increased titers and higher purity
  - Host cell proteins: characterization and selective removal *via* genetic modification
  - Continuous processing: *e.g.*, perfusion reactor; simulated moving bed
  - Non-chromatographic separations: *e.g.*, membrane-based purification, selective extraction/precipitation, magnetically-enhanced separations, self-processing proteins (such as intein) for enhanced separation
  - Process analytical technology (PAT)
- Single-use manufacturing
- Alternative production platforms
  - Accelerated production technology
  - Cell-free synthesis
  - Transgenic plants and animals

6.1 Biogenerics

Biogenerics – also termed biosimilars or follow-on biologics – are biotherapeutics that are designed to be "similar" in structure, biological properties and efficacy to their innovator counterparts whose patent protection has expired. The high degree of molecular complexity and heterogeneity of biologics in general, and therapeutic proteins in particular, makes it extremely difficult, if not impossible, to produce an exact copy of a marketed biologic using a non-identical manufacturing process.[30–32] Thus biogenerics represents a complex and controversial area with regards to economics, definition of equivalency, regulatory approval pathway,[33,34] safety and efficacy as well as social and commercial implications.[30,35] One trend is, however, quite clear. With the cost driven down by competition from biogenerics, manufacturing processes will have to become more efficient, with improvements likely coming from the new developers as well as the innovator companies themselves, some of whom already announced their intention to be key players in the biogenerics market such as Merck, Pfizer and Novartis.[36]

While still in its early stage with only a couple of similar biotherapeutics approved in Europe as of the end of 2009, the emergence of biogenerics represents a positive development in the long term from an environmental standpoint, since more cost-effective processes tend to use less materials and generate less waste. Additionally, for therapeutic proteins, whose production is typically burdened with complex downstream processing, the opportunity to develop integrated solution with an optimally redesigned upstream process – for instance, using a different host strain possessing higher fidelity for

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glycosylation,[36] or deleting genes encoding difficult-to-remove protein impurities[37] – will likely result in significantly more efficient manufacturing processes.

6.2 Process and analytical technologies

Intensive effort has been on-going to enhance process and analytical technologies for biologics manufacturing. The combination of cell line development and bioreactor optimization continues to push the titer up, from less than 1 g protein product per liter of cell culture broth in the early days to the current ~5 to 10 g per L or even higher,[25] with the resulting reduction in water and material usage. With rapid advances in genomics and proteomics, protein impurities from host cells, both microbial and mammalian, continue to be identified and characterized, helping to significantly simplify downstream processing.[37] Continuous processing is clearly an area of interest that has begun to make in-roads in various operations in the production of small-molecule drugs.[38–41] For protein therapeutics, perfusion bioreactors and simulated moving beds (or continuous chromatography) are two examples in this direction, with 60% or more reduction of buffer usage and several-fold increase in throughput compared to batch chromatography operation having been claimed for the latter.[42,43] A true continuous process for biologics, however, would have a major environmental impact through minimization of the control space (cleanroom) requirement, which substantially reduces energy and materials usage, as noted earlier.[27,28] Non-chromatographic separations such as membrane-based purification,[44,45] selective extraction[46,47] and precipitation,[48] magnetically-enhanced separations,[49] and the use of self-processing proteins (such as intein)[50] have the potential to significantly reduce the huge usage of water and chemicals associated with chromatography operations discussed in section 3.

Process analytical technology (PAT), initiated by the US FDA in 2004,[51] represents a "system for designing, analyzing, and controlling manufacturing through timely measurements (*i.e.*, during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality." In the implementation of PAT, physicochemical and biological methods are utilized in an integrated manner with mathematical and risk analysis tools. With numerous case studies demonstrating its utility in the traditional pharmaceutical area,[52,53] PAT has recently begun to be evaluated for implementation in the bioprocessing field, such as for control and monitoring of cell culture processes,[54–56] for product quality applications in vaccine production,[57] for pooling decision of chromatography column elution,[58] for reducing product variability in a diafiltration operation,[59] and for controlling protein refold.[60] While PAT clearly focuses on product quality, its methodology can have positive environmental consequences such as minimization of rejects, scrap, and re-processing; and facilitation of continuous processing that will improve energy and material use as well as increased plant capacity.

6.3 Single-use manufacturing

The adoption of single-use equipment, first with some specific units such as filters and membranes for operational convenience, has now spread to practically every operation used in biotherapeutics manufacturing, including solid and liquid transfers, mixing, solution preparation and storage, bioreaction, chromatography, tangential and normal flow filtration, and freezing. In addition, single-use equipment has been introduced to facilitate tasks such as aseptic and sterile connections, sampling, and process monitoring. The major limitation of single-use equipment is related to the material of construction – plastic – which results in reduced capabilities for operating pressure, heat transfer, mass transfer and scale up when compared with fixed stainless steel equipment. With single-use equipment up to 2000-L scale demonstrated to be capable of meeting biotherapeutic process requirements, production is now possible using all disposable equipment, called single-use manufacturing.

The primary drivers toward single-use manufacturing are speed to market to deliver therapeutic proteins to patients[61] and economics with the potential of lower upfront investment costs and cost of goods (COG).[62–64] Studies comparing a single-use plant *versus* a traditional one for mAb manufacture in terms of economics at scales ranging from 200 L to 2 × 5000 L and titers of 0.5 g per L to 2 g per L indicate that single-use plants have the potential to reduce capital investment requirements by 33–40% and COG by almost 20–30%.[62,63] The environmental impact has also been explored and, as expected, total water usage and chemical usage are found to reduce by half, primarily through less cleaning.[64] However, other analyses show significant variation in the savings depending on the specific application.[65] The economics varies significantly depending on where single-use components are used in the process and were found to be highly scale- and titer-dependent, with single-use advantages greatest at low titer and volume.[66]

Environmental assessment of single-use equipment takes a longer term and broader view than economic analysis. In the economic analysis at the 2 × 5000 L scale noted above,[64] the waste generated from disposable plastic bags increases by almost 170 kg per kg of protein for the single-use process over the traditional stainless steel one. Additionally, overall environmental assessment needs to take into account additional water and chemicals used by the equipment suppliers themselves in generating single-use equipment, including environmental contamination resulting from assembly and sterilization operations. Leveen and Cox carried the above analysis further taking into account energy consumption that included manufacture and transport of plastic bags.[67] Expressing the overall energy consumption as carbon footprint, they found interestingly that the single-use plant would use 35% less than the traditional steel plant.

Assessment of single-use environmental impact is further complicated by the multiple options for disposal: recycling, pyrolysis, incineration, incineration plus cogeneration, and landfill. Recycling has the least environmental impact but is complicated because many single-use components are constructed of multiple polymers. Plastic has a heat value 1.5 times that of coal so incineration plus cogeneration can reduce the environmental impact. However, many single-use components are simply incinerated or sent to a landfill because other options are not available or when the waste stream is not of sufficient magnitude.[68,69] Single-use environmental assessment should also include details such as sterilization techniques for single-use equipment, which can include small waste streams

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containing hazardous materials such as cobalt-60 for gamma radiation generation. A comprehensive evaluation of single-use technology thus requires understanding of the equipment, manufacturing and regulatory operations, economic analysis and environmental assessment.

6.4 Alternative production platforms

A more drastic change in the ways therapeutic proteins are produced involves a paradigm shift in either production technology or producing agents. Driven by the need for rapid response to biological warfare attacks, the Defense Advanced Research Projects Agency (DARPA) has initiated a program called "Accelerated Manufacturing of Pharmaceuticals" with the objective of producing target biologics, such as vaccines, not only with the required high quality but also fast and inexpensively.[70] Although highly ambitious, this program could provide the impetus needed for a major departure from conventional approach, encouraging important developments such as highly productive and robust microbial systems, flexible modular design of unit operations for rapid assembly and automation, continuous processing, and the use of transgenic plants (addressed in more detail below). Progresses achieved in this program will have the potential to greatly minimize resources required for production of biologics.

Alternative production platforms could involve neither microbial nor mammalian cells directly; these include cell-free synthesis,[71,72] transgenic plants,[73-77] and transgenic animals.[78-80] In cell-free synthesis, cells are grown primarily to harvest their metabolic and protein production machinery (for example, ribosomes, RNAs, enzymes, reducing and oxidizing factors) for "chemically" synthesizing the protein of interest from simple raw materials. Significant progress has been made with this production method, moving from simple proteins initially to complex entities such as antibody fragment bioconjugates and virus-like particles. Due to many similarities between the two production means, especially in downstream processing, the cell-free approach will unlikely result in major beneficial environmental impacts.

Plants genetically modified to express foreign proteins – called transgenic plants – have been developed to produce various protein products in many plant species, such as maize, rice, barley, alfalfa, and tobacco, to name a few. The production process in this case involves harvesting the plants and extracting the product from the collected biomass; the typical protein purification operation that follows is not unlike that for proteins produced by fermentation,[81-84] perhaps somewhat simpler. The actual synthesis part is obviously different, typically done in the field or in a greenhouse, requiring less complex facilities and operations compared to those associated with the traditional manufacture of therapeutic proteins. Thus protein production using transgenic plants can be less resource intensive and is claimed to have much better economics, with cost of goods at least an order of magnitude lower than traditional fermentation-based processes,[85] even though this estimate likely covers only the upstream (product formation) portion, which constitutes about 30% of the overall cost of goods that includes DSP, packaging, sales and distribution, *etc.*[88] Additionally, environmental containment of plant production systems, such as possible genetic exchange with unintended plants and other potential ecological effects, is a critical issue that needs to be properly addressed. Growing aquatic plants such as duckweed[86] in a bioreactor-like environment is closer to the traditional microbial or mammalian cell production methods. In this case, because of light requirement for growth and production, aquatic plants tend to grow near the surface where they are exposed to the light source and consequently do not utilize the full liquid volume in the reactor as microbial and mammalian cells do.

An intriguing emerging application of transgenic plants is in the production of vaccines by plants,[87] even in edible forms such as seeds and fruits creating "edible" or "oral" vaccines.[87-91] Compared to traditional vaccines, edible vaccines could offer much lower production cost, convenient storage and transportation (ambient temperature, no refrigeration), simplicity of use, economic administration, and mucosal immune response. However, important technical, logistic and especially regulatory problems remain to be addressed in order to make edible vaccines a reality.

Analogous to transgenic plants, transgenic animals are those genetically modified to produce foreign proteins, typically in their milk. This has been successfully demonstrated for over 20 different proteins in cows, goats, sheep, pigs, rabbits or mice,[92-95] and even in insects such as silk worms.[96] Transgenic animals appear particularly promising for the production of large amounts of therapeutic proteins including mAbs.[92,97] Specific examples include antithrombin III in goat milk for preventing blood clotting,[98] lysostaphin in mouse milk for preventing *Staphylococcus aureus* infection,[99] human growth hormone in the milk of guinea pigs.[100] While the upstream process (product synthesis) with transgenic animals is quite different to that of fermentation-based processes, downstream processing is similar but somewhat simpler.[84] Key technical issues facing the commercial production of therapeutic proteins using transgenic animals include efficiency and speed of producing a commercial product (genetic modification and breeding to establish the herd), effective handling of infectious diseases, and potential differences in post-translational characteristics compared to those produced by mammalian cell culture.[101-103]

7. Conclusion

The E factor for process water represents an appropriate environmental index for production of therapeutic proteins, since aqueous solutions are used in practically every processing step. Significant reduction in water usage along with chemicals and consumables could be achieved with process enhancement and simplification, but recycling of water and chemicals – rarely implemented in protein manufacturing due to lack of an economic driver – would result in an even greater impact and can play an important role if waste disposal becomes an issue. Useful environmental indices for biologics manufacturing should also include energy consumption, which is dominated by facility operations, especially cleanroom or controlled space because of the required HVAC for its operation. Thus a great deal of attention should be paid to improving non-process operations at bioprocessing plants due to their disproportionate usage of water and energy. Overall, an improved understanding of the use of energy and water in biotherapeutics manufacturing as well as innovative development to minimize the requirement

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of cleanroom space itself, such as closed operations routinely practised in other industries, could significantly reduce the environmental impact of facilities, especially at the earliest stages of architectural design. Of the emerging developments, biogenerics stands out as the major business driver forcing more efficient manufacturing. Single-use practice could have a significant impact on both materials and energy usage in manufacturing but for now is limited to relatively small-scale operations. The transgenic approach appears to hold promise in the long term for a drastic shift in protein production practice, but with potentially very complex environmental implications that need to be worked out over time.

Acknowledgements

The authors would like to thank the following colleagues in Pfizer Biotherapeutics Pharmaceutical Sciences for their helpful input and discussions: Andrew C. Espenschied, Robert E. Kottmeier, James F. Bouressa, Ferhana Zaman, Arin Bose and Michelle Wang. We also appreciated inputs from Brad Storrs and Greg Gibb of Monsanto, Suzanne S. Farid and James Pollock of University College London, Brian Kelley of Wyeth (now with Genentech), and Lindsay Leveen of Genentech.

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Downloaded by City College of New York on 24 November 2010
Published on 12 April 2010 on http://pubs.rsc.org | doi:10.1039/B927443J

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