Bioburden: current innovations and practices to address microbial contamination in downstream bioprocessing

Bioburden: current innovations and practices to address microbial contamination in downstream bioprocessing

Bioburden control is an area of serious concern for both manufacturers of biologicals and suppliers to the industry. This white paper considers some of the risks related to downstream processing and presents recent developments by suppliers that help manufacturers mitigate these risks. Topics covered include improvements in raw material quality, equipment design, chromatography resin properties, and ways of working. Challenges specifically related to the sanitization of protein A chromatography resins are also discussed.

Bioburden control in biopharmaceutical production

The risk of microbial contamination is inherent in most production scenarios for biologicals. Conditions in the upstream process, with controlled temperature and a nutrient-rich medium, support not only growth of the target cells but also of other biological organisms.

Downstream operations also have their share of challenges. Open containers may be used to transfer product or handle buffers. Raw materials and processing aids that have contact with the product, like chromatography resins and buffer components, are introduced. The transfer of equipment between rooms exposes the process to contamination. Unit operations like chromatography frequently involve a large amount of manual handling, for example during resin slurry preparation and column packing. Success is highly dependent on the skill and experience of personnel; and wherever humans intervene there is a potential source of bioburden.

In this white paper, we will discuss opportunities and challenges related to bioburden with a focus on downstream bioprocessing.

The potential impact of bioburden

Regulatory authorities set high standards regarding bioburden control due to the potential impact on public health (1,2,3). Concerns relate not only to the risk of product contamination with microorganisms but also to effects caused by released toxins, as well as possible influences on product stability and potency. In addition to potentially tragic effects on patients, the economic impact of batch failure due to a bioburden incident is enormous. For mAb blockbusters, a month's production stop would result in lost revenues of up to 1 billion USD, as well as considerable costs associated with investigating root causes and sanitization in the production facility (Table 1). Damages due to patient law suits and long-term loss of reputation are more difficult to estimate, but can be significant.

Addressing the challenges of bioburden requires vigilance on the part of the drug manufacturer, but suppliers to the industry also play an important role. This can be related to raw material quality assurance, new ways of working and product innovations that allow maintenance of high standards of hygiene or enable better sanitization. This white paper reviews some recent contributions to the field of bioburden control.

Table 1. Potential impact and costs of a bioburden incident in biopharmaceutical manufacturing

Issue	Potential impact and cost
Commercial impact	Up to USD 1 billion in lost revenue[1] - Loss of reputation by customers, authorities, patients - Long lead time due to low inventory - Lost business to competitors - Penalties in rare cases
Failed production lot/scrap batch	Up to USD 1 million[2]
QA investigation	Up to USD 20 000[3]
Sanitization of facility and equipment	Up to USD 100 000[4]
Resin must be discarded	Up to USD 3 million[5]

Assumptions:

1. Up to USD 1 billion lost revenues/month for blockbuster drugs
2. 2000 L bioreactor with 5 g/L expression level. Cost of mAb production USD 100/g
3. USD 120/h labor cost. 1 week of investigation by three people
4. USD 120/h labor cost. 4 weeks by five people
5. Based on large-scale column size and resin costs

Understanding bioburden

Potential microbial contaminants can take many forms, including bacteria, viruses, and molds. In addition, there are risks of exotoxin or endotoxin contamination.

An understanding of the properties of these various potential contaminants can help us mitigate risks. This knowledge has led to the establishment of sodium hydroxide (NaOH) as the cleaning and sanitization agent of choice for many re-used process components. However, there are challenges with the use of NaOH. For example, some re-usable materials are not resistant to high concentrations of NaOH. Also, spore-forming bacteria, like Gram-positive *Bacillus subtilis* (*B. subtilis*), are difficult to get rid of because of their resistance to even high concentrations of NaOH. Residual spores can propagate subsequently under favorable conditions, for example during re-equilibration and loading of rich harvest material onto a chromatography column.

Endotoxins are heat-stable lipopolysaccharides associated with the outer membranes of Gram-negative bacteria, such as *Brucella, Neisseria*, and *Vibrio* species, as well as *Eschericia coli*. Endotoxins are released when the bacterial cells are disrupted. In large quantities, they produce hemorrhagic shock and severe diarrhea. Smaller amounts cause fever, altered resistance to bacterial infection, leukopenia followed by leukocytosis, and other biologic effects. Sodium hydroxide solutions (> 0.1 M) are often used to inactivate endotoxins.

Exotoxins are soluble, antigenic, usually heat labile, injurious substances elaborated by certain Gram-positive or Gram-negative bacteria. They are formed within the bacterial cell, but released into the surrounding environment where they are highly potent. Most exotoxins are proteinaceous with a wide range of molecular weights. The toxic effects of the molecule are destroyed by heat, prolonged storage, or chemicals, leaving a toxoid substance. There are also peptide-based exotoxins, which tend to be more resistant.

Vegetative bacteria are those bacteria or microorganisms that are growing and reproducing, and not dormant or forming spores. They can be removed by a range of procedures including filtration, heat, and treatment with a range of chemical agents.

Spore forming bacteria can form spores, which allow the organism to survive in hostile environmental conditions. Bacterial spores are made of a tough outer layer of keratin that is resistant to chemicals, staining and heat. The spores allow bacteria to remain dormant for years. This protects them from various traumas, including temperature differences and the absence of air, water, and nutrients. When conditions become favorable they are able to transform to a vegetative state and propagate. Spore forming bacteria cause several diseases, including botulism, anthrax, tetanus and acute food poisoning. Spores are difficult remove but are susceptible to heating above 121°C and exposure to oxidative agents such as sodium hypochlorite and peracetic acid solutions.

Viruses are infectious agents that replicate inside the cells of other organisms including animals, plants and bacteria. In general, virus particles are much smaller than bacteria and consist of a nucleic acid core surrounded by a protein capsid. Some virus particles also have a lipid envelope, making them susceptible to treatment with solvents and detergents. Most virus particles can be removed by filtration although smaller ones, like parvoviruses, are more difficult. A wide range of human diseases are caused by viruses, from the common cold and influenza, through to AIDS and Ebola.

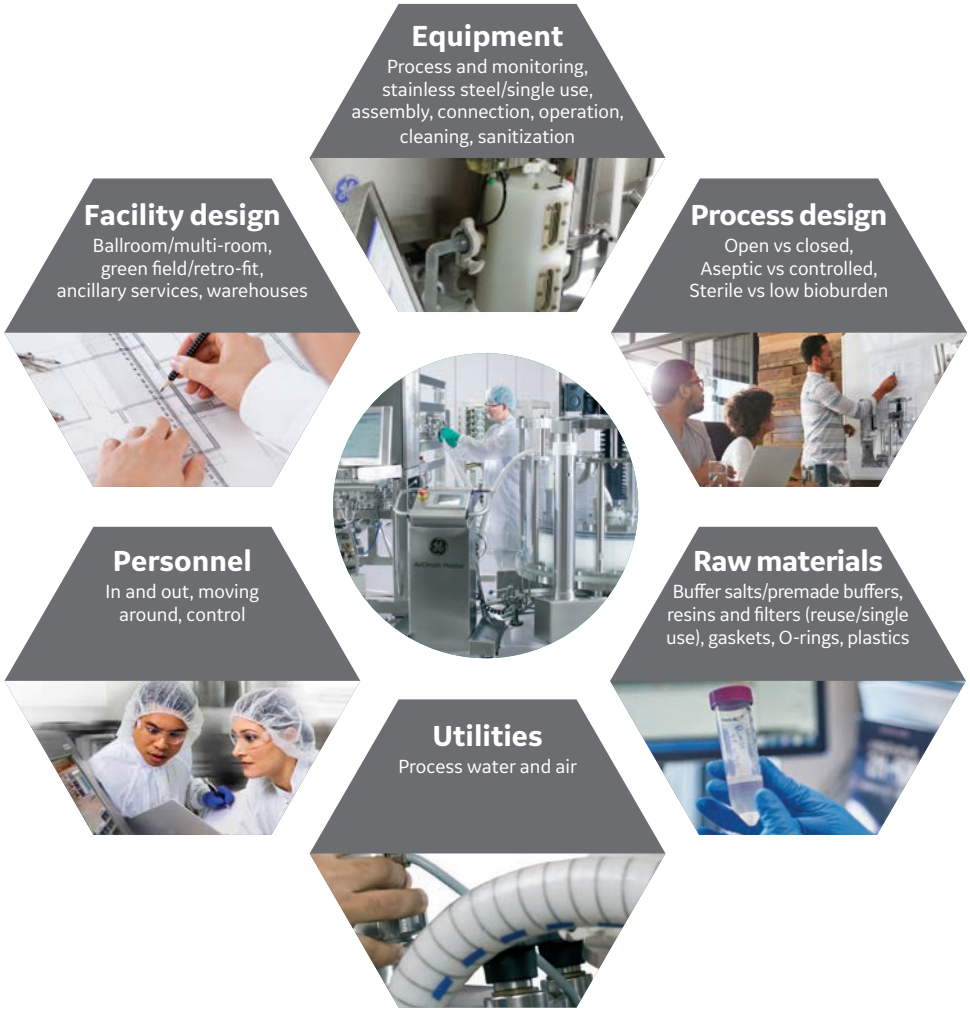

Fig 1. Sources of bioburden in biomanufacturing. Adapted from Reference 4.

Sources of bioburden

Bacteria, fungi and viruses are present in water, soil, air, plants, animals, and humans. They can enter a process through many routes. Bioburden control involves measures to prevent entry of such contaminants into the production process, process steps that remove them from the product (e.g. filtration, chromatography, low pH inactivation, solvent-detergent treatment), and sanitization activities.

As a process manager, you are concerned about bioburden throughout the lifecycle of the product, from designing the manufacturing process and a new facility to producing the last commercial batch after many years of product supply. You may have a wide range of alternatives to choose from, or you may be limited to small tweaks and changes because of the existing infrastructure of the facility. If you are designing your process from scratch, you have a major opportunity to apply a strategy for facility and process design to help you avoid potential sources of bioburden (Fig 1).

There are many considerations that depend on the design of the facility. Clearly, retro-fitting a process into an existing building poses constraints not found in a ”green field” project. Furthermore, there are widely differing approaches to constructing new facilities, for example, there are some designs with many rooms and others dominated by a single large room. The choice can be built as prefabricated units or using more traditional construction methods (Fig 2).

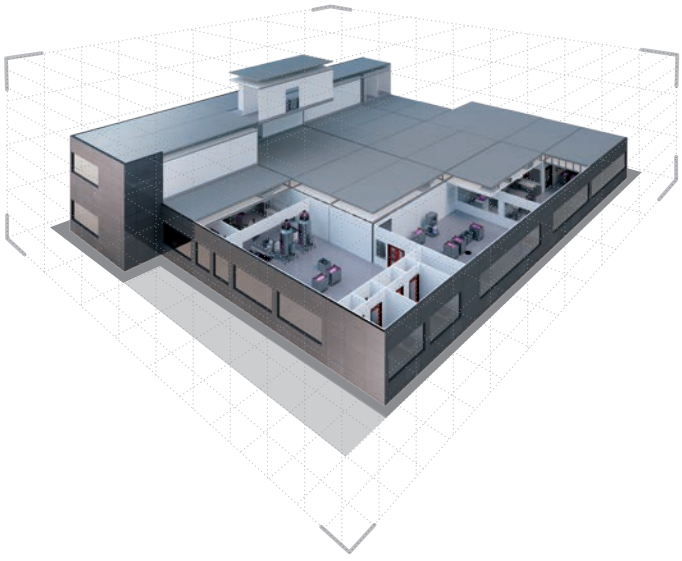

Fig 2. Prefabricated KUBio™ facilities use primarily single-use technologies for manufacturing mAbs.

Options for addressing bioburden

Bioburden in the production facility will be highly dependent on the choice of equipment–fundamentally whether single-use or stainless steel is preferred, but also related to detailed design features. This will have an impact on contamination risks as well as on the need for cleaning and sanitization and the scope and scale of sanitization utilities. Procedures for handling the equipment need to address specific bioburden issues and staff need proper training to mitigate risks. Process monitoring also introduces important potential routes of microbial contamination that need to be considered when choosing equipment, or documenting procedures and training personnel.

Process design, including types of unit operations involved, will influence to what extent closed systems, single-use equipment and automation can be used. Raw materials, including filters, chromatography resins, and buffer components, are other potential sources of microbial contamination, as are utilities like process water and air flow into the facility. Finally, personnel in the production facility pose a major bioburden risk that must be addressed by procedures (like gowning), training, the use of closed systems, and automation (5).

Bioburden control is a huge area that must not take second place to other production concerns. It should be prioritized and addressed by the drug manufacturers and their suppliers together, with constant dialogue to ensure mutual understanding of situations, risks and capabilities.

Raw materials

Raw materials and processing aids that have direct contact with the product molecule during its production are potential sources of microbial contamination. Clearly chromatography resins fall into this category and benefit from appropriate quality assurance measures by the supplier. Normally a biopharmaceutical manufacturer will audit a supplier and these meetings become an ideal forum to address quality issues.

For more than 30 years, resin production at GE Healthcare has been the subject of regular quality audits by customers. Bioburden has been a frequent area for discussion which has resulted in a range of improvements that includes better cleaning methods, extensive training of operators, regular monitoring of process water, improved monitoring of microorganisms and particles in sieving and filling areas, shorter hold times, and reduced open procedures.

These improvements have resulted in very encouraging QC test results. For example, 94% of MabSelect SuRe™ resin lots released between January 2010 and May 2017 have shown no detectable bioburden (Fig 3).

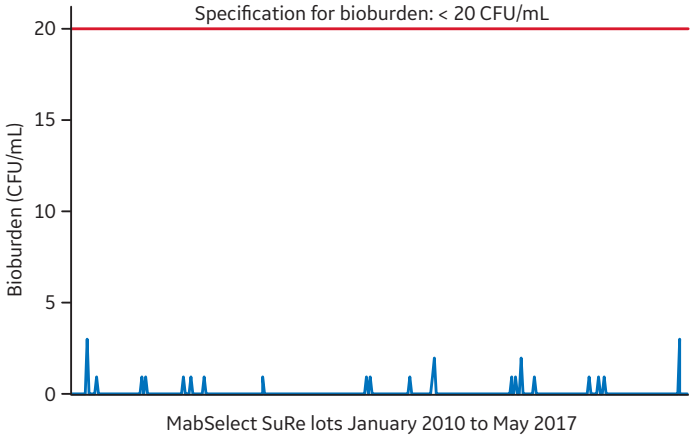

Fig 3. Impact of bioburden control on chromatography resin microbial test results.

Recently, the microorganism specification for selected protein A resins and Capto™ resins was decreased five-fold from less than 100 colony-forming units (CFU)/mL to less than 20 CFU/mL. A specification for endotoxins (< 5 endotoxin units (EU)/mL) has also been introduced.

Preventing bioburden in production operations

Single-use solutions

The introduction of single-use solutions has in many ways simplified bioburden control, at least at smaller scales. These single-use, prepacked products are usually delivered presanitized. Today, cell culture can be performed in bags and upstream and downstream operations can be done in the same "ballroom"-style facility with high bioburden control and great flexibility and adaptability as products, scales of production and unit operations change over time (6).

Column operation and closed handling

Another development with positive impact on bioburden control is automation. For example, automated solutions now exist that address the challenging workflow of column packing, operation, and unpacking (5). The whole process becomes much less "hands-on", more reproducible, and essentially a closed operation, apart from the initial transfer of resin from its transport container (Fig 4). Automated column handling combined with innovative design features allow high standards of hygiene.

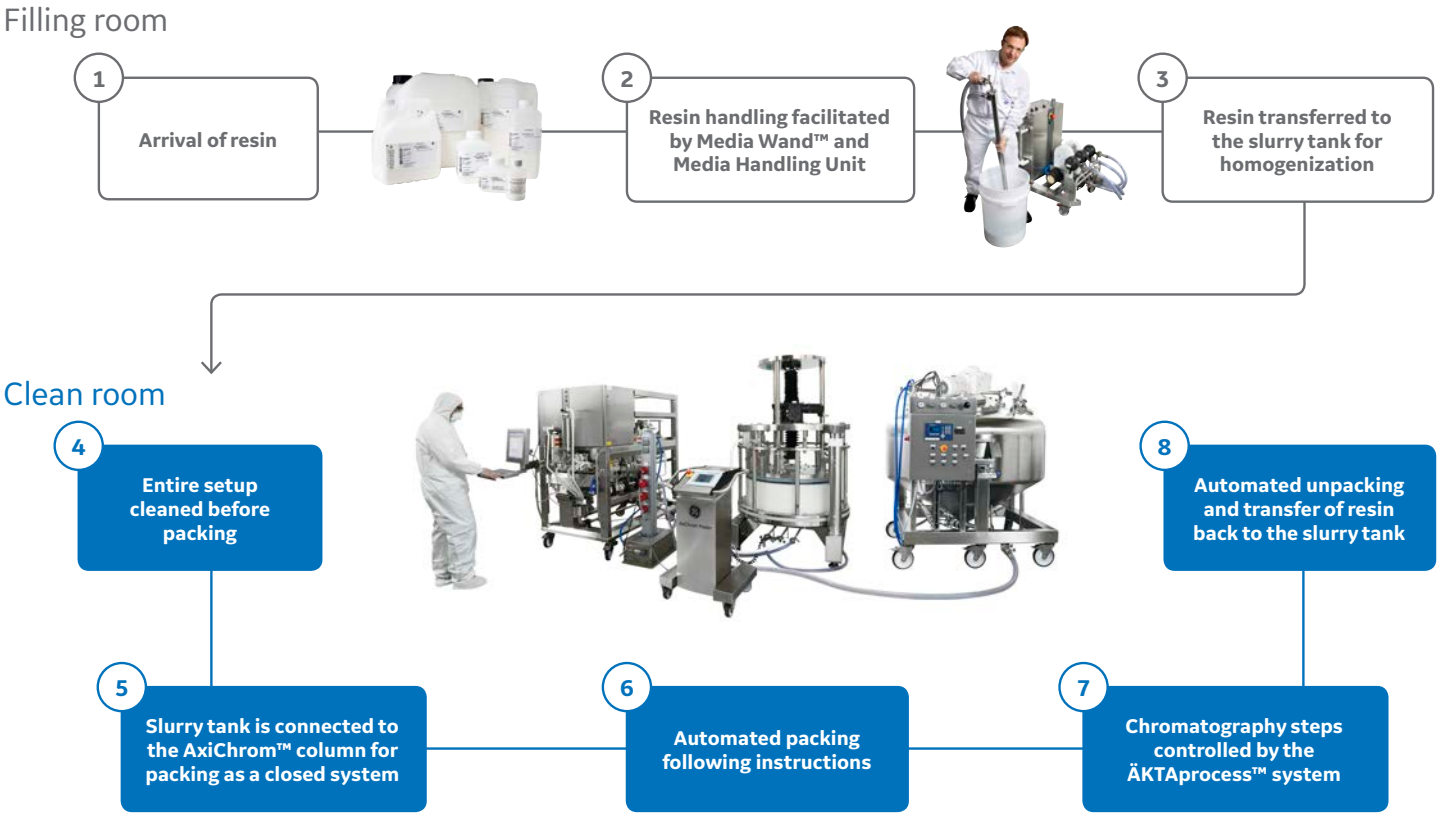

Fig 4. Column packing in a closed system means manual handling of resin and buffers is kept to a minimum. This is achieved by connecting the column, slurry tank and chromatography equipment in a closed system (blue boxes).

Continuous and connected bioprocessing

Developments in continuous bioprocessing and connected unit operations potentially allow the use of closed systems. A move in this direction is encouraged by the biopharmaceutical industry, partly for reasons of process economy, and by regulatory agencies for reasons of product quality (7).

Closed and automated systems help reduce the exposure to bioburden. With connected unit operations, hold times can be reduced and open tanks are usually avoided. However, a potential problem is that continuous operations can be online for days, weeks, or months instead of hours. The time for bioburden to flourish increases—so the operation must consider raising the bar even higher to prevent contamination.

Buffer preparation

The volume and number of buffers for a typical downstream process is often considerable. Manual preparation of these buffers is a potential risk for bioburden entry. Automated buffer preparation, creating buffers in-line, at point of use, is available, and clearly minimize manual operations (5). With in-line conditioning, buffers are prepared from concentrated stock solutions of acidic and basic buffer components and water for injection (WFI). In-line conditioning simplifies buffer preparation and significantly reduces the number and volume of buffer tanks as well as the floor space needed. It is a more accurate and reproducible way to prepare buffers than simple dilution. Furthermore, premade stock solutions, sterile filtered into gamma-irradiated single-use bags, are available to further streamline buffer preparation and provide opportunities for better control of bioburden control (Fig 5).

Fig 5. In-line conditioning from HyClone™ buffer stock solutions and WFI reduces manual operations and exposure to contamination

Storage

Storage of equipment and consumables also introduces a risk for microbial growth. It is therefore important to sanitize equipment and chromatography resins before storage. Chromatography resins should be stored in bacteriostatic solutions. There are a number of demands on these solutions that need to be fulfilled. They should be non-toxic to humans, inexpensive, and easy to dispose of. A crucial property is the stability of the chromatography resin in the solution. The resin should be stable not only for short periods in between use, but for its entire shelf life (8).

Cleaning and sanitization

Cleaning and sanitization are necessary procedures in a production process where unit operations, like chromatography, are re-used over many cycles. Experience has resulted in NaOH becoming the backbone of most sanitization and cleaning-in-place procedures, due to its efficacy, low cost, and ease of detection, removal, and disposal. Apart from its ability to inactivate endotoxins and many microorganisms, it can remove bound proteins, nucleic acids and lipids from the chromatography resins making it an excellent cleaning and sanitization agent for chromatography resins. In industry, it is common to use 1 M NaOH, when possible, for cleaning and sanitization of chromatography columns and resins. For chromatography resins with protein ligands that are sensitive to high alkaline concentrations, lower concentrations of NaOH or alternative cleaning and sanitization solutions have to be used. Data on the effects different concentrations of NaOH on microorganisms, viruses and endotoxins are shown in Table 2 and Figures 6 and 7 (9).

Cleaning: chemical solubilization and physical removal of soil, organic debris, and particulates from surfaces

Sanitization: the use of any chemical agent to reduce a microbiological population (endotoxin, virus) to an acceptable, predetermined level

Table 2. Microorganism inactivation with NaOH

Organism	NaOH (M)	Time[1]	Temp (°C)
E. coli	0.01	2 h	4 or 22
S. aureus	0.1	1 h	4 or 22
C. albicans	0.5	1 h	4 or 22
A. niger	0.5	1 h	4 or 22
P. aeruginosa	0.5	1 h	22
B. subtilis spores	1.0	48 h[2]	22
B. subtilis spores	1.0	8 days[3]	4

[1] For reduction to below detection limit < 3 organisms/mL
[2] For reduction to below detection limit < 10 organisms/mL
[3] For reduction to below detection limit < 100 organisms/mL

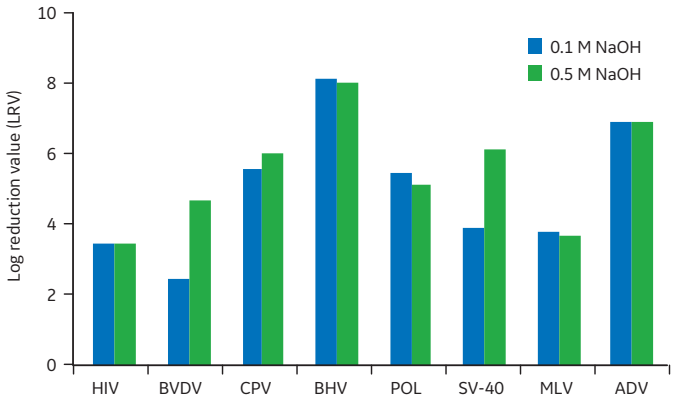

Fig 6. Viral inactivation with NaOH. ADV = human adenovirus type 2, BHV = bovine herpesvirus type 1, BVDV = bovine viral diarrhea virus, CPV = canine parvovirus, HIV = human immunodeficiency virus type 1, MLV = murine leukemia virus, POL = human polio virus type 2, SV-40 = simian virus 40.

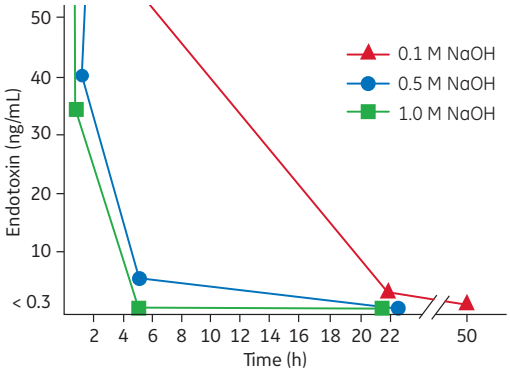

Fig 7. Endotoxin inactivation with NaOH .

Design and sanitization of chromatography columns

Whilst column designs have improved with respect to handling and operation, sanitization has always remained an important consideration. Success will depend upon the design of the column and the power of the sanitizing solutions. In particular, dead spaces that might reduce cleaning efficiency need to be avoided. Sanitization studies by column suppliers are useful to help biological manufacturers establish their own sanitization procedures (Fig 8) (10).

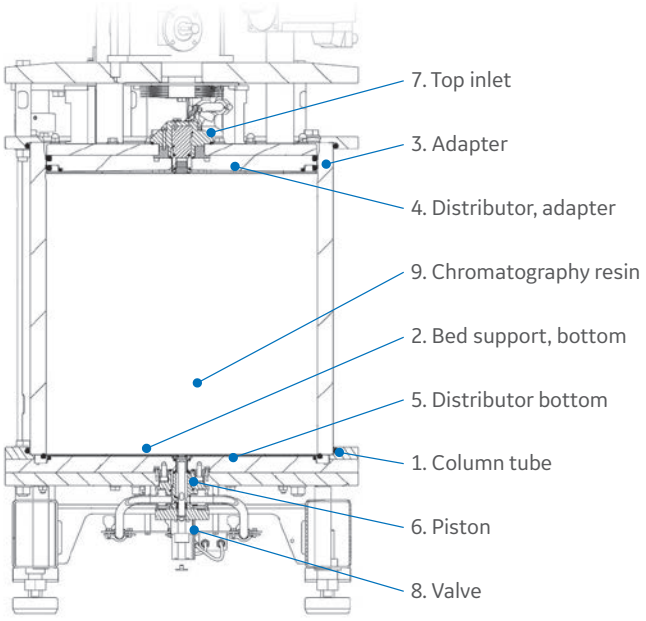

Fig 8. Sanitization of AxiChrom columns. 53 sites were sampled on an AxiChrom 600. The column was packed with Sepharose™ Fast Flow resin and challenged with *E. coli* ATCC 8739 (10^6 viable organisms/mL). There were no surviving *E. coli* after sanitization with 1.0 M NaOH for 5 h. The endotoxin concentration was less than 0.25 EU/mL (USP recommendation for WFI).

Challenges in the sanitization of chromatography resins

Probably the most well-known problem regarding cleaning and sanitization of chromatography resins is the sensitivity of protein ligands to high concentrations of NaOH. In particular protein A resins, widely used in platform processes for mAbs, are affected. Protein A resins are used in the capture step of mAb downstream purification where cell culture nutrients and other impurities are at their highest levels and a highly efficient cleaning and sanitization method is most desirable. This has led to efforts to improve the resistance of protein A resins. Figure 9 shows that good performance is maintained following repeated cleaning/sanitization cycles with 0.5 M NaOH for two chromatography resins that contain protein A ligands that have been stabilized by genetic engineering.

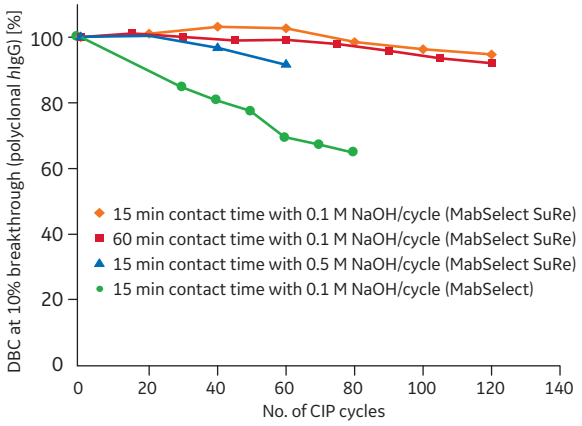

Fig 9. Dynamic binding capacities of MabSelect SuRe and MabSelect resins for polyclonal human IgG after CIP with 0.1 or 0.5 M NaOH for up to 200 cycles (9).

Ongoing developments, including continued engineering of the protein A ligand, indicate that further improvement in alkali stability is possible (Fig 10).

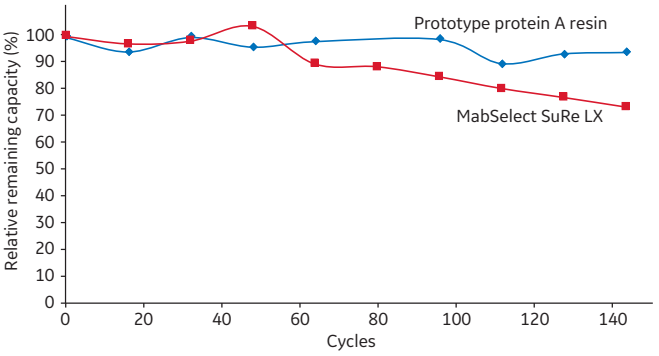

Fig 10. Protein A resins packed in columns and used in an accelerated study with exposure to 0.5 M NaOH for 36 hours, corresponding to 144 15-min cycles of 0.5 M NaOH followed by IgG binding tests.

Spore-forming bacteria and sanitization of chromatography resins

As mentioned earlier, some potential microbial contaminants are resistant to cleaning and sanitization with NaOH. Finding methods that are sporicidal and at the same time compatible with chromatography resins and wetted material in hardware or single-use components, is another challenge in bioburden management. In tests with the protein A resin, MabSelect SuRe, concentrated NaOH is not sufficient for complete spore reduction even after an extended contact time of 24 h (Fig 11). A mixture of 1.0 M NaOH and 2% benzyl alcohol gives slightly improved inactivation and a mixture of 1.0 M NaOH and 40% 2-propanol gives more than 6-log reduction at 4 h contact time. However, organic solvents are not a preferred option for large-scale manufacturing and none of these conditions are compatible with today's protein A resins. Studies have shown that the oxidizing agent peracetic acid (PAA) is much more efficient for reduction of bacterial spores when compared with NaOH. More than a 6-log reduction of *B. subtilis* spores was obtained within 30 min when using 20 mM PAA (11).

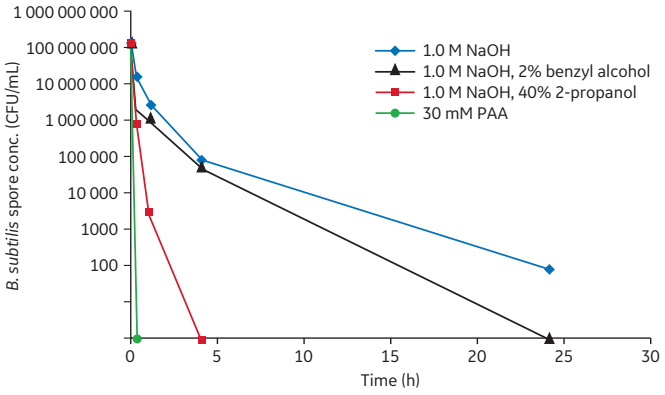

Fig 11. *B. subtilis* spore kill kinetics of various sanitization agents in 50% MabSelect SuRe resin slurry. 30 and 20 mM PAA is sporicidal with a contact time of 15 to 30 min.

PAA is a strong oxidizing agent which might be expected to have negative effects on the performance of chromatography resins, in particular those containing protein ligands. However, studies show that limited contact with PAA (two to three times during the lifetime of MabSelect SuRe) (can be used to provide a good safeguard against spore-forming bacteria, without having an unacceptable impact on the resin performance and lifetime (12).

Conclusions

Bioburden control can be tackled in several ways and every manufacturer of biologicals has its own set of challenges. Suppliers to the industry also have an important role to play, which is most productive when there is a good dialogue, mutual understanding of risks and capabilities, and frequent collaboration. The drive towards single-use solutions, efforts to improve quality standards in raw materials, and developments in closed and automated systems are examples that can help manufacturers mitigate risks of microbial contamination. Improvement of sanitization and cleaning methods is an important focus, not least regarding protein A resins that are key to most mAb production processes. Development of more alkali-stable protein A resins is in progress opening possibilities for more rigorous cleaning and sanitization procedures.

References

1. Current good manufacturing practices for finished pharmaceuticals, 21 CFR 211. www.fda.gov/ohrms/dockets/98fr/98110s1.pdf
2. Biological product general 21 CFR 600-610. www.ecfr.gov/cgi-bin/text-idx?SID=36ed3d89fc7bd2191036894b12b7ca50&mc=true&tpl=/ecfrbrowse/Title21/21cfrv7_02.tpl#0
3. Good manufacturing practice guide for active pharmaceutical ingredients, ICH Q7. www.fda.gov/downloads/Drugs/.../Guidances/ucm073497.pdf
4. Suvarna, K. *et al.* Case studies of microbial contamination in biologic product manufacturing. *Am. Pharm. Rev.* **14(1),** 50–56 (2011).
5. White paper: Unlocking the potential for efficiency in downstream bioprocesses. GE Healthcare, 29195721, Edition AB (2016).
6. Poster: ReadyToProcess increases facility capacity and shortens change-over time. GE Healthcare, 28946087, Edition AA (2008).
7. Farid, S. *et al.* Continuous bioprocessing: the real thing this time? *MAbs* Vol. **6**, 1357–1361 (2014).
8. Application note: Use of benzyl alcohol as a shipping and storage solution for chromatography media. GE Healthcare, 28989901, Edition AC (2014).
9. Application note: Use of sodium hydroxide for cleaning and sanitization of chromatography media and systems. GE Healthcare, 18112457, Edition AI (2014).
10. Application note: Sanitization and endotoxin clearance in AxiChrom columns. GE Healthcare, 28929042, Edition AE (2014).
11. Monie, E.M. *et al.* Sanitization method for affinity chromatography matrices. World Intellectual, Property Organization. Publication no. WO2016139128A1. 9 Sep (2016).
12. Poster: Collaborating to address the bioburden challenge. GE Healthcare, 29254080, Edition AA (2017).

GE Healthcare Bio-Sciences AB
Björkgatan 30
SE-751 84 Uppsala
Sweden

gelifesciences.com/bioprocess

GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK
GE Healthcare Europe GmbH, Munzinger Strasse 5, D-79111 Freiburg, Germany
GE Healthcare Bio-Sciences Corp., 100 Results Way, Marlborough, MA 01752, USA
GE Healthcare Dharmacon Inc., 2650 Crescent Dr, Lafayette, CO 80026, USA
HyClone Laboratories Inc., 925 W 1800 S, Logan, UT 84321, USA
GE Healthcare Japan Corp., Sanken Bldg., 3-25-1, Hyakunincho Shinjuku-ku, Tokyo 169-0073, Japan
For local office contact information, visit gelifesciences.com/contact.

29265476AA 06/2017