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3

Aseptic Processing of Protein Pharmaceuticals

Michael Townsend

1. INTRODUCTION

The objective of this chapter is to present a planning guide for designing and implementing aseptic processing for drug product manufacturing, for both clinical supplies and the commercial market, from the perspective of the unique characteristics of protein-containing drug products. The discussion will be restricted to solution, suspension, and lyophilized dosage forms in final containers made of glass or plastic. It will present a conceptual framework for the development of aseptic processes at the lab bench, subsequent scale-up to clinical batches, and, finally, validation of the fully scaled-up and well-defined processes for commercial manufacturing of the drug product.

2. ASEPTIC PROCESSING OF SOLUTION, LYOPHILIZED, AND SUSPENSION DOSAGE FORMS

The processes necessary to transform a pharmacologically active protein or peptide into a safe, efficacious, and reliable parenteral drug

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Development and Manufacture of Protein Pharmaceuticals, edited by Nail and Akers. Kluwer Academic/Plenum Publishers, New York, 2002.

product must first be adequately developed and validated to reproducibly yield product that meets specifications. These processes must then be carried out in controlled areas on suitably qualified equipment by trained personnel using standard operating procedures and manufacturing instructions to document the operation. Most of the processes in sterile pharmaceutical manufacturing must be done aseptically so that the final product can be guaranteed to be nonpyrogenic and have a sterility assurance level (SAL) of 1×10^{-3} or less by the end of solution or suspension filling, or lyophilization, if applicable.

The SAL of an aseptic filling process refers to the probability of a nonsterile vial being produced during the aseptic operations. It is an empirically derived value, which is determined by the results of media fills. A media fill entails the compounding, sterile filtration, and filling of microbiological growth media as simulated product into vials (to include the lyophilization step, if applicable), followed by incubation of the filled vials to detect the presence of microbiological contamination. At a minimum, it is recommended that at least 3000 vials be filled for each test. The ratio of the number of contaminated vials to the total number filled gives the SAL (i.e., to achieve an SAL of 1×10^{-3} no more than 3 vials may contain microbial growth for every 3000 vials filled). Such media fills must be repeated on a regular schedule to revalidate the aseptic operations. In addition to media fills, other microbiological control measures must be employed, such as periodic environmental filter inspections, routine microbiological monitoring of the manufacturing personnel and environment, and routine air quality monitoring in the sterile core (see Section 2.1.4) (USP, 2000a, pp. 2099–2106; 2000b, p. 2147).

The U.S. Food and Drug Administration (FDA) has proposed that terminal sterilization should be employed as an adjunct to aseptic processing to further increase a parenteral product's SAL (U.S. Food and Drug Administration [FDA], 1991). However, most protein or peptide drug products do not possess sufficient heat stability to allow for the use of typical high-temperature sterilizing methods (autoclaving or dry heat), and the use of ionizing radiation (gamma, E-beam, or X-ray) can also result in unacceptable levels of physical and chemical degradation (Yamamoto, 1992). Therefore, aseptic processing is of fundamental importance in preventing microbial contamination of a protein-containing formulation during drug product manufacturing.

The term "aseptic processing" implies a number of fundamental requirements: (1) All product contact equipment and final packaging components must be sterilized using validated cycles, (2) the processes must be carried out in environmentally controlled facilities under Class 100

high-efficiency particulate air (HEPA)-filtered,* laminar flow air, (3) the operators must be properly trained in aseptic processing, and (4) the operators must be gowned in attire to prevent the shedding of viable and nonviable particulates into the work area. In a restrictive sense, aseptic processing begins with the sterile filtration of the formulated bulk solutions and continues through to the sealing of the closures in the primary containers. In a broader sense, though, all of the parenteral product manufacturing facilities, utilities, and support functions that supply raw materials, equipment, components, and formulated bulk solutions to the these downstream operations fall under the "aseptic processing" umbrella. These functions play a critical role in achieving the desired SAL and preventing pyrogenic contamination of the final product containers produced in a parenteral drug product manufacturing operation.

2.1. Current Aseptic Processing

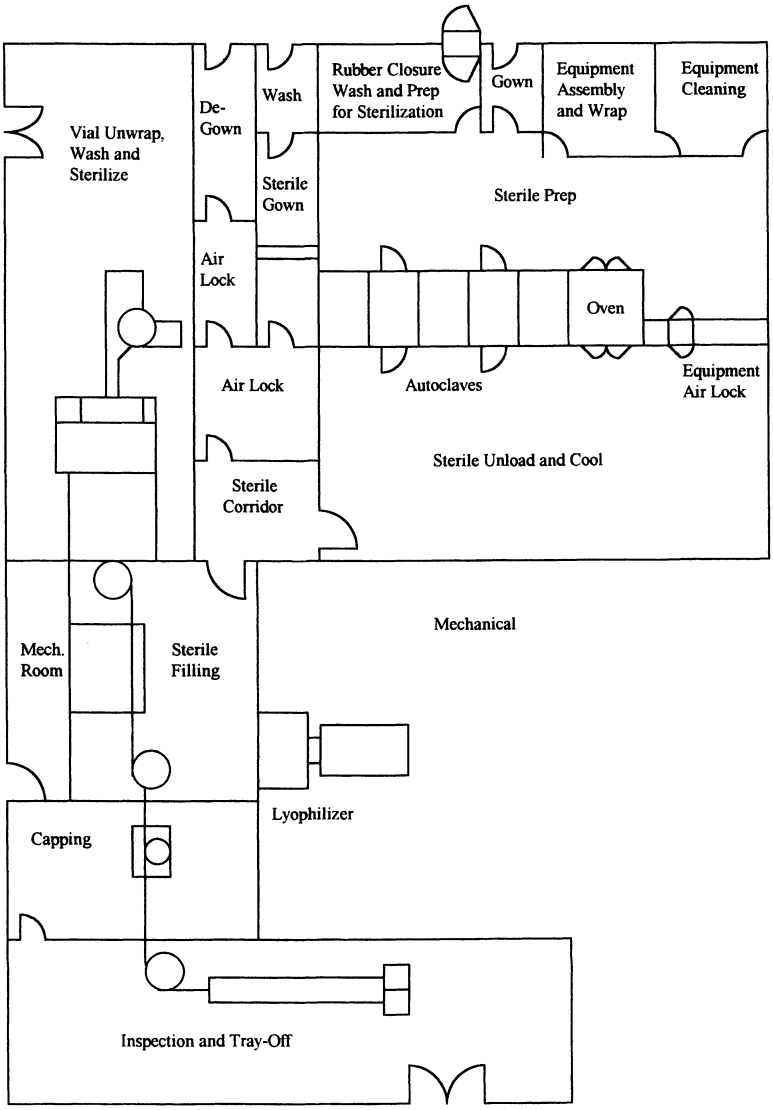
Descriptions of current practices in aseptic processing are available in a number of guidelines, articles and books (Center for Drug Evaluation and Research, 1987; (Akers, 1988; Akers *et al.*, 1988; Center for Drug Evaluation and Research, 1987; Fry, 1987; Hofmann, 1993; Trappler, 1993), so this section will not devote significant space to the subject. However, many of the requirements necessary for successful aseptic processing will be reviewed here so as to furnish the reader with a baseline of information.

2.1.1. FACILITIES

The facilities that house an aseptic processing area must be logically planned so as to facilitate the unidirectional flow of components, equipment, bulk product, and filled/sealed product containers through the area. An example of such a facility is shown in Fig. 1. The design should provide an increasing level of cleanliness as the process moves toward the final aseptic filling, lyophilization, and sealing area(s). The heightened levels of cleanliness are achieved by increasing air quality levels, controlling access to the areas (airlocks and gowning areas), and increasing the degree to which the personnel are gowned.

The manufacturing areas should be easily cleaned (i.e., seamless ceilings and walls, and nonporous floors with coved molding) and large enough to allow for the proper orientation of permanent equipment (e.g., filling lines,

*A HEPA filter is an extended-medium dry-type filter in a rigid frame having a minimum particle collection efficiency of 99.97% for particles having diameters > 0.3 μm.



lyophilizers) and unencumbered movement of personnel and portable equipment (e.g., tanks, pumps, tray racks, etc.) around these permanent pieces of equipment. The materials of construction should be non- or low-particle shedding and impervious to aggressive cleaning and disinfecting solutions.

2.1.2. UTILITIES

Water, compressed gases, clean steam, and heat, ventilation, and air conditioning (HVAC) services must all meet the current good manufacturing practice (cGMP) regulations for parenteral drug product processing (U.S. FDA, 1996). These include descriptions of the types of materials used for construction, required operating specifications, equipment design, and requirements for controlling and testing for microbiological and extraneous chemical contamination.

The quality of water to be used in each of the various processes within the manufacturing area is dictated by cGMP regulations:

- Water for injection (WFI), defined as distilled or reverse osmosis water kept at 80°C in a recirculating loop or batched on a 24-hr schedule, must be used as the final rinse water for all product contact equipment and final product containers and closures. It must also be the water used as a raw material for compounding formulated bulk drug product.

Figure 1. A modern parenteral processing area consists of zones of different degrees of cleanliness. This is designed to act as a barrier to the movement of viable and nonviable contamination from noncontrolled areas into the critical areas where sterile products are filled, and lyophilized, if applicable. The levels of cleanliness are specified in U.S. Federal Standard 209D (Clean Room and Work Station Requirements, Controlled Environment, 15 June 1988), and are defined by the number of particles greater than or equal to specified diameters allowed per cubic foot of air. A diagram of a parenteral facility is shown. The "sterile filling room" is the most critical area, where sterile products and sterilized equipment/components are manipulated while being exposed to the environment. An air cleanliness level of at least Class 100 (i.e. NMT 100 particles $\geqslant 0.5$ μm, NMT 300 particles $\geqslant 0.3$ μm, and NMT 750 particles $\geqslant 0.2$ μm) must be maintained in this area. Rooms in which sterile products are not exposed to the environment, but through which sterilized components must move, like the sterile corridor, and sterile unload and cool areas, are designed to meet less stringent air cleanliness levels (generally Class 10,000). Separate in-going and out-going air locks and gown/degown rooms act as buffers to further protect the cleanest areas. HEPA-filtered air supply, high air flows, and pressure differentials moving from the cleanest to less clean areas, as well as special room finishes, all aid in achieving the environment necessary for parenteral manufacturing.

- Demineralized water, generated either by ion exchange methods and/or reverse osmosis, may be used as process wash or rinse water prior to a final rinse by WFI, when applicable.

Compressed gases used in the aseptic operation that could potentially contact sterile drug product or equipment to be used in its processing must be free of particulate contamination, both viable and nonviable, and must be free of extraneous chemical contamination, for example, compressor oil.

Clean steam, generated from WFI, must be used in the autoclaves or sterilize-in-place (SIP) systems that sterilize product contact equipment.

HVAC systems that support the aseptic manufacturing areas must be designed to prevent microbiological contamination (especially fungal) and maintain low, controlled relative humidity levels during the extremes of outside weather conditions.

2.1.3. PARENTERAL MANUFACTURING

A process flow chart for the manufacturing of a typical lyophilized drug product is shown in Fig. 2. For comparison, a flow chart for a suspension drug product is shown in Fig. 3.

2.1.3.1. Preparation of Product Contact Equipment

Preparation of product contact equipment is identical for all three of the dosage forms. Dirty equipment is disassembled and certain items, such as silicone transfer tubing, are generally discarded. Potentially disposable items such as transfer tubing are often viewed as single-use components, because of the difficulty and cost of developing and validating suitable cleaning procedures. Stainless steel and reusable polymeric parts, for example, gaskets and pump diaphragms, are cleaned in a suitable washing device, such as a high-agitation water recirculation bath, a cabinet spray washer, or an ultrasonic bath, using a validated cycle. After the final WFI rinse, the equipment is dried with filtered compressed air and stored in closed stainless steel containers in a Class 10,000 room. When the clean equipment is needed to manufacture a batch of a protein product it is assembled in a Class 10,000 room, wrapped in particle-free paper or placed in a stainless steel–covered box, and then sterilized in a double door autoclave opening into the aseptic processing suite. Compounding and receiving tanks may be cleaned manually or by an automated clean-in-place (CIP) system. Cleaned tanks

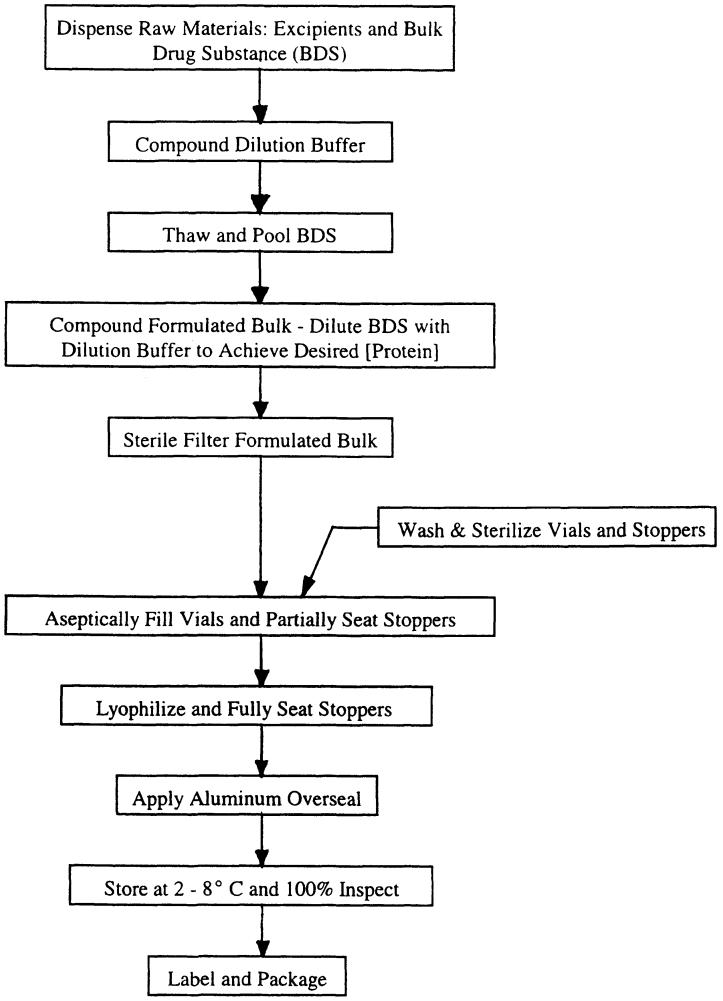


Figure 2. Flow chart of lyophilized drug product manufacturing process when the API is supplied as a frozen solution.

intended as sterile reservoirs for filter-sterilized, formulated bulk drug product may be autoclave sterilized or sterilized-in-place using high-pressure, saturated steam delivered directly into the tank.

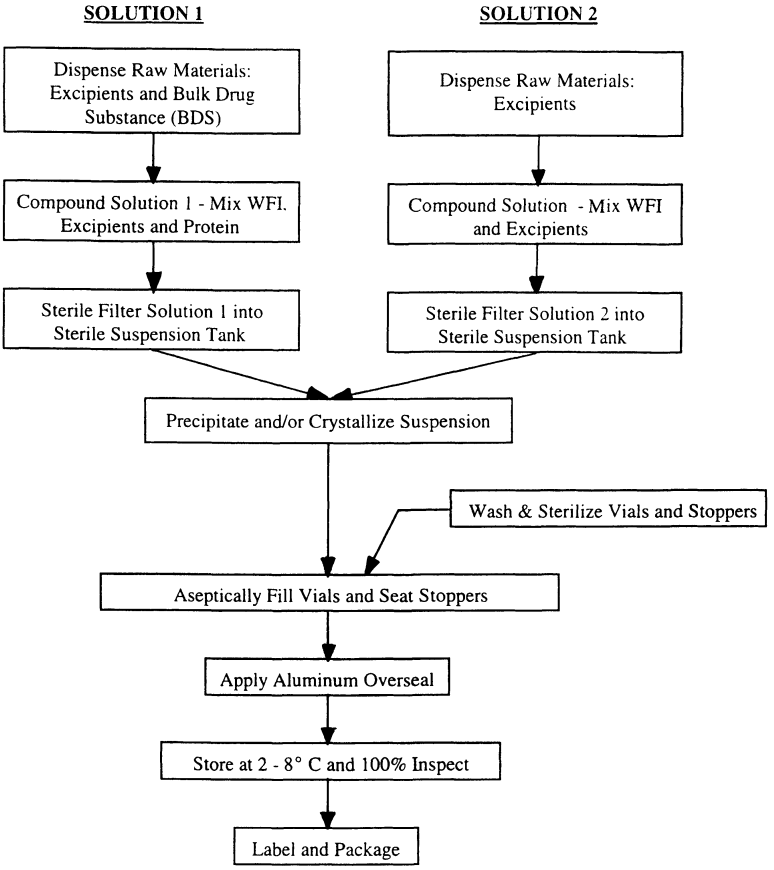


Figure 3. Flow chart of a suspension drug product manufacturing process.

Sterilization and depyrogenation of product contact equipment and packaging components must be accomplished prior to their entry into the aseptic processing area for use in the filling operation. Fundamental aspects of thermal sterilization processes are covered in Chapter 4 of this volume, and there are numerous, more detailed documents describing development and validation of sterilization processes (Parenteral Drug Association [PDA], 1978, 1981, 1996). Depyrogenation can be achieved by thermal destruction or by the removal of pyrogenic materials (bacterial endotoxins)

by washing and/or rinsing. Validation of depyrogenation is accomplished by demonstrating that a process can reduce a spiked bacterial endotoxin challenge of at least 1000 USP units to no more than 1/1000 of the starting quantity, that is, a three-log cycle reduction (USP, 2000c, pp. 2144–2145). Many of the component parts of product contact equipment cannot withstand the high temperatures necessary for depyrogenation, which for practical purposes must be greater than 170°C (Nakata, 1994, Tjusi and Harrison, 1978). Therefore, depyrogenation is accomplished by a combination of appropriate cleaning and a final WFI rinse. Subsequent sterilization is carried out using saturated steam, either in an autoclave or via an SIP system.

2.1.3.2. Component Preparation

Preparation of packaging components is also identical for all three of the dosage forms.

(a) Glass containers (vials, syringes, or ampoules) are washed in either a fully automated or semiautomatic washer that employs multiple water and air injections at differing temperatures to help clean the vials by thermally shocking them. The removal of glass spicules (small glass particles, sometimes partially fused to the vial wall, left in the containers as a result of the vial-making process) from the interior of the glass containers is best accomplished by using an ultrasonic bath prior to the water and air blasts. The final WFI rinse occurs immediately before a siliconization step, if it is required for syringe barrels or as an antiadsorptive coating.

(b) The glass containers may be batch-sterilized and depyrogenated in hot air convection ovens at temperatures of 200–250°C, or in a continuous-process, tunnel oven at approximately 300°C through which the containers pass on a mesh, metal belt. In both cases, the ovens are supplied with HEPA-filtered, Class 100 air. Heating of the glass containers in a tunnel oven occurs either via direct radiation from infrared heating elements or convection from heated air that is forced through high-temperature HEPA filters located immediately over the glass containers on the moving belt.

(c) Rubber closures are prepared by washing in a suitable washing device, for example, a sparging overflow kettle, or a fluidized bed washer. In many cases, rinsing with heated WFI along with air sparging to generate agitation will optimally clean rubber closures. The use of detergents or other cleaning agents during washing to facilitate removal of leachables, manufacturing residues, or particulate matter must be carefully evaluated during the development of the closure cleaning cycle. Many of the newer rubber formulations currently used for closures contain little or no undefined filler

material that could be a source of leachables. However, if cleaning agents must be employed, there is an added burden on the rinse portion of the cycle to assure the reduction of these potential extractables (i.e., the cleaning agents) to levels that would not adversely affect the stability of the drug product formulation.

(d) If siliconization of rubber stoppers is required, and it is in most cases, in order to achieve filling line machinability and prevent clumping during autoclaving, it may be carried out in the same vessel in which the washing took place or the closures may be transferred to another piece of equipment for this operation. Silicone may be added as a dispersion in water or as an emulsion, which contains emulsifying agents. Closures are then placed in either stainless steel trays with lids or in combination Tyvek/heat-resistant plastic autoclave bags and sterilized in a double-door autoclave opening into the aseptic processing suite. As with the product contact equipment, depyrogenation of closures is accomplished using optimized washing techniques and a final WFI rinse.

2.1.3.3. Compounding

The manufacturing differences for the three dosage forms (solution, lyophilized, and suspension) first appear at the compounding stage. For a solution or lyophilized product, the bulk protein, either as a dried powder or as thawed frozen protein concentrate, is mixed with the excipient solution. The resulting solution is sterile-filtered into a sterilized receiving vessel prior to filling. Parenteral suspension dosage forms are produced in two ways: (1) by aseptically dispersing a sterile powder of known particle-size distribution into a sterile liquid medium in which it is insoluble or (2) by combining two sterile solutions which bring about the formation of an amorphous or crystalline solid phase *in situ* (M.J. Akers *et al.*, 1987). The sterile powder referred to in method 1 may be formed by bulk lyophilization and milling to achieve a uniform particle distribution, by precipitation or recrystallization, or by spray drying. An oil-based suspension of recombinant bovine somatotropin is an example of a protein suspension prepared by this method. For method 2, multiple solutions must be compounded, sterile-filtered, and then aseptically mixed to generate the suspension solid phase. Examples of protein product suspensions manufactured in such a manner are insulin zinc suspensions and isophane zinc insulin suspensions.

For a suspension generated by combining two filter-sterilized solutions (method 2 above), if the solid phase is to be crystalline, a hold time may be required to allow for crystallization to take place. Crystallization may occur

de novo or through an intermediate amorphous precipitate, which generates the microenvironment necessary for crystallization to be initiated and then propagated. Examples of critical parameters in this step may be the temperatures of the solutions, the agitation rates, and the hold times. A terminating solution may be added to the crystalline-growing medium to end the crystal growth process and stabilize the crystals once the desired particle size has been achieved.

2.1.3.4. Filling

Filling of solution and lyophilized dosage forms can be carried out on virtually any of the currently available filling machines, for example, positive-displacement syringe filler, rolling-diaphragm pump filler, or time-pressure filler. The different types must be evaluated for their compatibility with the protein product in question. Filling rates, needle sizes, pump sizes, the need for bottom-up filling, and the manner of supplying an inert gas for an oxygen-sensitive product must all be part of the process development work. In order to assure elegant dried cakes, lyophilized formulations must be filled in such a manner as to avoid splashing of product onto the walls or neck area of the vial or foam formation during filling.

For oxygen-sensitive products, an inert gas such as nitrogen may be introduced to replace the air in the headspace of compounding and sterile holding tanks as well as the final product containers. On the filling line this may be accomplished by flushing the vial with nitrogen from a delivery needle prior to, during, and/or after solution filling. The nitrogen must be sterile-filtered prior to its introduction into the tanks or vials to prevent viable and nonviable particulate contamination. The gas pressure, flow rate, and purge time must be optimized during process development so as to meet the oxygen tension specifications necessary to prevent oxidative degradation of the product. Closure insertion and capping immediately after filling/nitrogen purge should prevent air contamination.

Filling of suspension dosage forms requires that the solid phase be maintained homogeneously distributed throughout the suspending medium during the entire operation. This can be accomplished by using a suitable sterile holding tank mixer, for example, a VIBRO-Mixer or a magnetically coupled mixer (see Section 3.1.1.4 for a description of these mixers), and a recirculation system which transports the bulk suspension formulation from the holding tank to a manifold, from which the filling pumps are fed, and then back to the holding tank. A peristaltic pump can be used to power the recirculation system, because of its suitability for a closed, aseptic operation. The recirculation system not only must transport the formulation to the filling machine, but also maintain the

homogeneity of the suspension during its transit. Mixing during recirculation can be achieved by the maintenance of turbulent flow in the system. The diameter of the tubing (or piping) and the pump speed will affect the amount of turbulence or mixing to which the suspension is subjected during recirculation. The recirculation system must be free of dead ends and irregular curved or kinked paths at which suspension particles could collect leading to heterogeneity. The sedimentation properties of the suspension particles in the formulation will determine the degree of mixing turbulence required to maintain homogeneity from the holding tank into the vial.

The design of the recirculation system must take into account the viscosity of the suspension formulation, the particle size and density of the suspension, the morphology of the particles (globular, needle-like, etc.), and the fragility of the suspended particles. All mixing and recirculation parameters must be optimized to balance the requirement for maintenance of suspension homogeneity with the necessity to avoid damaging or altering the suspension particle size or morphology. All mixing and recirculation systems impart varying degrees of stress to the formulation, mostly in the form of shear stress to the suspension particles. Long exposures may result in significant abrasion or breakage of the suspension particles, potentially altering their dissolution characteristics. Therefore, the recirculation system design should be optimized to avoid suspension particle damage for the maximum time period during which the suspension might be exposed during a filling operation.

2.1.3.5. Lyophilization

Lyophilization is an important unit operation for production of either final pharmaceutical product or a process intermediate, where it may be necessary to concentrate the protein or to store it in a relatively non-growth-promotive state. Aseptic processing considerations apply primarily to production of finished, sterile lyophilized products, and is the focus of this discussion.

Lyophilization takes place in chambers incorporating sanitary design principles. Current FDA guidelines require sterilization of the chamber/condenser after each batch of final pharmaceutical product. Newer lyophilizers have CIP and SIP capabilities, which greatly increases the assurance of achieving reproducible levels of cleanliness and desired probabilities of sterility.

A lyophilizer chamber and condenser leak test should be performed prior to each cycle to assure that the sterility of the product load will not be compromised due to an adventitious leak. This can be done with a vacuum hold test, which can be run and evaluated by an automated lyophilizer

controller. If all of the volatile materials have been removed from the chamber before the test, there should be minimal pressure rise observed during the vacuum hold period (Trappler, 1993).

Lyophilization trays that must be transported outside of HEPA-filtered, laminar flow protection before loading into the freeze-dryer chamber should be equipped with covers to protect the partially stoppered vials from particulate contamination. Tray covers are removed immediately before loading the trays into the chamber. The entrances to the lyophilization chambers should be protected by HEPA-filtered, laminar flow air to protect open vials during the loading phase. Trays of product can be loaded into the lyophilizer either manually or using automated tray loaders, usually onto shelves that have been preset to the initial temperature for the cycle, for example, approximately + 5°C. It is desirable to remove tray bottoms after loading to achieve better and more uniform heat transfer from the shelves to the vials. For bottomless trays, rectangular rings usually remain around the vials from each tray to maintain tray identity and to aid in replacing the tray bottom for unloading following cycle completion.

In order to monitor the temperature profile of the product in the vials during lyophilization, small thermocouples are usually placed in vials prior to the initiation of the cycle. Probed vials, generally at least three, are placed in trays at different locations within the lyophilizer load, for example, on the top, middle, and bottom shelves of the dryer. Thermocouples should be located in the same position within each vial—in the center of the vial and touching the bottom—because this is the location where the last ice sublimes during drying. Special thermocouple holders are available to facilitate precise placement (see Fig. 2 in Chapter 6 of this volume). The thermocouple wires should be of large enough gauge (small enough diameter) so as not to add significant mass or volume to the filled solution, which may alter its freezing or drying characteristics.

Placement of thermocouples in the vials, by necessity a manual operation, poses a risk to sterility assurance, because of the proximity of the operator and his or her activities to other open vials in the load. This raises the issue of where the probed vials should be located in a tray and also at what position on the lyophilizer shelf. The thermocouple wire, initially sterile before being handled by the operator while being located in the vial, can pose a potential hazard of both viable and nonviable particulate contamination to open vials over which it may be placed. Because of lateral heat transfer from the walls and door of the chamber and the resulting "edge effect" causing faster drying of vials at the edge of the shelves, the most representative location for thermocoupled vials is in the center of a tray at the center of a shelf. However, some manufacturers believe that the risk to the product in terms of decreased sterility assurance outweighs the benefit of monitoring the most representative vials. Therefore, many manufacturers choose to place probed vials at the front edges of trays

located closest to the lyophilizer door, where the risk to product sterility is minimized. The probed vials are then discarded after the completion of lyophilization.

In addition to the trade-off between process monitoring considerations and sterility assurance aspects of the process, formulation scientists and process development personnel should recognize that temperature data from thermocoupled vials are biased relative to the rest of the batch. This arises because the thermocouple acts as a seed site for heterogeneous nucleation of ice crystallization. As a result, monitored vials undergo less supercooling, freeze slower, have a larger average ice crystallite size, and dry faster than nonmonitored vials. This bias must be taken into account when determining freezing, primary drying, and secondary drying times for initial cycle development as well as during scale-up. These points underscore the need for improved process monitoring technology that does not depend upon monitoring of individual vials. This point is discussed further in Chapter 6.

The vacuum level in the chamber and condenser should be controlled automatically during the cycle by the addition of either dry, sterile-filtered air or nitrogen. A capacitance manometer should be used to measure the vacuum level for both monitoring and controlling purposes. Vacuum level in combination with the shelf temperature and the inherent heat and mass transfer characteristics of the frozen and partially dried cake affect the product temperature of the frozen solution in the vials. The product temperature must be maintained below thermal transition events above which meltback or collapse of the cake may occur, for example, eutectic temperature (T_e) or collapse temperature (T_g'). These thermal transitions are characterized during formulation and lyophilization cycle development using a variety of techniques, such as thermal analysis and lyomicroscopy. The cycles developed to accommodate these critical parameters must be checked out at both lab and commercial scale to evaluate the ability of a scaled-up lyophilizer to achieve adequate temperature and vacuum control to prevent collapse.

After the completion of the lyophilization cycle, stoppering of the vials is done while the vials are still in the chamber and after the required headspace environment and pressure have been established. Products that are sensitive to oxygen could have dry, filtered nitrogen introduced into the chamber or a high level of vacuum could be maintained, for example, 100- to 200-μm Hg vacuum. In most cases stoppers are seated in the vials with at least a partial vacuum remaining in the vial, for example, approximately 1 psig chamber pressure, so as to assist in maintaining container/closure seal integrity during subsequent processing and storage. Stoppering takes place by compressing the shelves together with a

mechanism that is completely within the lyophilizer chamber so as to avoid the potential for external contamination of the load. Properly siliconized stoppers are a necessity to assure that they will easily slide into the vial neck and seat fully in the vials, and then will not pop out of the vial after the shelf pressure is removed.

2.1.3.6. Capping

Assuring that the contents of the vial remains sterile requires the application of an aluminum overseal to keep the stopper properly seated in the vial and to maintain container/closure seal integrity. This operation, commonly termed "capping," should be done in a protected environment. Until the vials are capped they are still vulnerable to inadvertent contamination, because there is nothing in place to firmly compress the stopper flange onto the vial crown to form a secure seal. Capping is generally done in a room immediately adjacent to the aseptic filling room with an HEPA-filtered, laminar flow hood covering the capping machine and the conveyor transporting the vials out of the aseptic area and into the capper. Cappers are known to generate microscopic aluminum particles during their operation, which is the main reason that this operation is performed outside of the aseptic area. Container/closure seal integrity depends on the application of adequate seal compression force on the stopper. This important parameter can be monitored by utilizing a device to automatically measure the forces applied during the sealing process on each vial. This attachment to the capper, such as a seal force monitor (e.g., Genesis Machinery Products, Inc., Exton, PA), will monitor sealing forces and reject vials to which insufficient compression force has been applied during the sealing process.

2.1.3.7. Inspection

All parenteral product lots are 100% inspected after the completion of the capping operation and before labeling takes place. For solution products the objectives of the inspection are to determine (1) if the container/closure/seal system is defective in some way, for example, a vial missing a closure, cracked glass container, or inappropriately crimped aluminum overseal, and (2) if the vial contents contain particles of foreign matter. Evaluation of solution color and clarity are also part of an inspector's charge during the inspection process. Product containers that are defective are removed from the batch and rejected. A tabulation of the numbers and types of defects is

maintained for each batch of product inspected. This information is critical in determining the final disposition of the batch by the quality control group.

For protein and peptide solution products, the inherent aggregation characteristics of the molecules may result in a decrease in solution clarity and the appearance of opalescence. The *European Pharmacopoeia* addresses the issue of small, uniformly suspended particles in a product container by requiring an objective evaluation of solution clarity measured in reference to standards (European Pharmacopoeia, 1997). Eckhardt *et al.* (1994) describe the development of a spectrophotometric assay that can be correlated with the opalescence reference standards, effectively eliminating the need for their repeated preparation. Such an analytical tool may aid an inspector in estimating the degree of solution opalescence or haziness during an acceptability evaluation.

Inspection of lyophilized products includes the same container/closure defect evaluation as described above, but the examination of the vial contents focuses on the consistency and appearance of the lyophilized cake. Lyophilization should yield a cake that retains the same volume and shape as the starting solution with no evidence of collapse, melting, or foaming. Partial collapse is often observed in freeze-dried cakes of protein formulations, at least in part because of low collapse temperatures of some protective solutes (see Chapter 6). If protein stability is significantly lower in a partially collapsed matrix, then collapse becomes more than a pharmaceutical elegance issue: Areas of local collapse which go undetected could result in subpotent product vials prior to the expiration date. For this reason, it is good practice to carry out special stability studies on vials culled from scale-up or production batches which contain partially collapsed cakes. The stability of the protein in these cakes is then compared with that of normal cakes. If the stability in a partially collapsed matrix is not significantly different from that of noncollapsed cakes, then these data can support the position that collapse is a cosmetic defect, with more liberal inspection acceptance criteria.

Inspection of a suspension product focuses primarily on defects in the container/closure system. This is because the product suspension particles prevent detection of contaminating particles or fibers, unless they are visually distinguishable from the product suspension particles.

Visual inspection of solution product containers may be conducted by trained and qualified inspectors, automated equipment that incorporates an image analysis system for detecting defects, or a combination of both. Human inspections must be carried out in specially lighted enclosures having both a light and a dark background against which the product

containers are viewed. Groves (1993) presented a comprehensive description of the procedures, operator training, and equipment that is used for visual inspection of parenteral product containers.

Automated equipment must be calibrated using particles of known sizes seeded into the product solution, and then the detection process in the product containers must be validated. Knapp and Abramson (1990) published a performance-based validation procedure for automated particle inspection systems that is applicable to a wide variety of machines.

Swirling or other types of agitation are used in both human and machine inspections to suspend contaminating particles so they can be detected. For protein or peptide products, agitation employed during the inspection process may have to be minimized, because many proteins and peptides are sensitive to interfacial denaturation that may be induced by such movements (see Section 3.1.1).

Inspection of lyophilized and suspension product containers can only be conducted by trained and qualified human inspectors. Automated systems do not have the capability, at this time, of evaluating the quality of lyophilized cakes. Suspension products are opaque, or at best transluscent, which would also preclude an automated inspection process.

2.1.4. MEDIA FILLS AND ENVIRONMENTAL MONITORING

Media fills, air filter certifications, and routine environmental monitoring of the aseptic processing area and personnel are all part of the evaluation plan for assuring that aseptic operations meet predetermined specifications and yield products having a high level of sterility assurance. Media fills are an integral part of the validation (and revalidation) of aseptic filling operations. Suitable microbial growth media, for example, tryptic soy broth, is used as a surrogate for the drug product formulation, so that any microorganisms that may contaminate a vial during processing would be detected by the appearance of microbial growth and turbidity in the vial contents after appropriate incubation times and temperatures. Aseptic filling processes must demonstrate an SAL of 1×10^{-3} or lower.

Media fills that simulate the inclusion of lyophilization in the manufacturing process must also attain this same SAL. FDA guidelines indicate that only certain aspects of the lyophilization operation should be validated and thus mimicked in a media fill (U.S. FDA, 1993a). These include the transportation of the filled and partially stoppered vials to the lyophilizer, the loading of the trays of vials into the lyophilizer, and the full insertion of the stoppers into the vials, which occurs at the end of a cycle.

The guidelines suggest that neither freezing nor drying should be carried out after loading the media vials, because those steps may invalidate the results by killing some contaminating microbes or by drying the growth medium and making it less supportive of microbial growth. The sterilization of the lyophilization chamber and the gas backfill system must be validated separately, and is not the objective of the media fills.

Maintaining a suitable aseptic environment in which sterile drug products can be manufactured requires a well-defined plan for routinely evaluating the viable and nonviable particulate levels within the aseptic processing area (Munson and Sorensen, 1990). This includes the use of air particle monitors to quantitate total airborne particulates and either active or passive microbial monitoring methods. Microbial monitoring of the air is conducted via passive settling plates which contain suitable agar growth media and by devices that actively draw air in and capture viable microorganisms. The three types of active devices are liquid impingement, membrane filtration, and agar impaction. Surfaces within the aseptic processing area are also sampled to evaluate microbial levels. Microbial counts per unit surface area are obtained by using contact agar plates or swabbing a specified area as defined by a template.

Monitoring the aseptic operator's gowns and gloves for microbial contamination using agar contact plates is also routinely performed to assure that proper gowning techniques and aseptic procedures are being used in the aseptic area. Personnel monitoring is critical because it is well known that the major source of viable particulates in an aseptic processing area is of human origin.

2.2. The Future of Aseptic Processing

It is well accepted now that the next quantum leap in parenteral processing will be achieved by the optimization and commercialization of barrier or isolation technology as a substitute for conventional clean room operations (Haas, 1995). Several manufacturers are currently marketing their versions of such a technology (e.g., Bosch/TL Systems, LaCahlene, Despatch). There are potentially two main benefits to be gained from its use: (1) an economic benefit, because the expensive design, construction, and operation of typical clean room suites is not required for the use of this technology and (2) a potential for greater sterility assurance, because humans are effectively removed from the environment in which all aseptic operations are performed.

One objective of barrier technology is to enclose the aseptic operations so as to isolate them from humans, because particles shed from humans,

both viable and nonviable, are the major source of contamination in a clean room. At the same time, a barrier system should still allow for indirect human intervention for machine setup, remedying machine interruptions, and repair. This objective is being addressed in several ways by different suppliers of barrier technology systems. All of them share one common characteristic, though, minimization and enclosure of the controlled, ultraclean environment in which aseptic processing is carried out. All of the designs employ a system for controlled entry where equipment and supplies being moved into or out of the enclosure must be either chemically or thermally sterilized so that the sterility assurance within the barrier can be maintained to levels $<1\times10^{-3}$. In fact, some suppliers of barrier systems are claiming sterility assurance levels as high as 1×10^{-5}. Indirect human access is achieved by the use of plastic half-body ports or rubber glove ports. The equipment and surfaces within the barrier are chemically sterilized and the barrier is supplied with HEPA or ultra low penetration air (ULPA)-filtered* air to minimize particulate contamination of the filled drug product. The residual levels of the chemical sterilants used in both the main enclosure and the airlocks in these barrier systems must be evaluated for their potential to adversely affect the protein and peptide products that may be processed within them. This topic will be discussed in more detail in Section 3.6.

A barrier system could be designed that isolates virtually the entire parenteral manufacturing operation from beginning to end. Such a system would require detailed design and engineering to overcome the difficulties of materiel movement between different pieces of processing equipment with only minimal human assistance. This would be exceptionally challenging for a lyophilized product, where the traying of vials prior to loading of the lyophilizer would require sophisticated robotic tray handlers and loaders. In addition, to maintain the operational readiness and high production output expected of commercial facilities, such a design would have to address the difficulties of equipment changeovers in a limited access enclosure between filling operations.

CIP and SIP systems could be employed to clean and sterilize much of the equipment within the barrier. For use with macromolecular drug products there are cautions that must be heeded in the development of such systems, which will be discussed in Section 3.5.

Another approach might be to bring already cleaned, assembled, and wrapped changeover equipment into the barrier system through a direct connection to a specially designed autoclave. A double-door autoclave with its outlet linked directly to the barrier would avoid the need for time-

*An ULPA filter is an extended-medium dry-type filter in a rigid frame having a minimum particle collection efficiency of 99.999% for particle diameters >0.12 μm.

consuming movement of equipment through airlocks, which requires a chemical sterilization step. The dissipation of heat emanating from the autoclave door during and after the cycles would be engineered into the design. Clean, siliconized stoppers could be prepared outside the barrier and placed into stainless steel containers or sealed Tyvek/plastic bags, which could be sterilized in this autoclave and unloaded directly into the barrier for use in a filling order. Specially designed docking ports through which materiel, such as sterilized stoppers, could be passed into the barrier are another option to be considered in the design. Vials would be washed automatically and then passed onto a belt to carry them through a tunnel oven for sterilization and depyrogenation. The exit from the tunnel oven would open directly into the barrier system and feed the filler/stoppering machine. Such a system has been designed and is shown in Fig. 4.

The interior surfaces of a barrier system are sterilized on a routine basis using chemical sterilants, most of which are vapor-phase systems using agents such as hydrogen peroxide, steam/hydrogen peroxide mix, or peracetic acid (Davenport, 1989; Lysfjord *et al.*, 1995). One of the difficulties with chemical sterilants is the time necessary to carry out a typical cycle, which includes both a gas exposure phase and a subsequent evacuation or degas phase. Vapor-phase hydrogen peroxide mixed with steam has been shown to be an effective sterilizing agent with relatively short cycle times (Lysfjord *et al.*, 1995).

HEPA- or ULPA-filtered air within the barrier is used to maintain a positive pressure differential and protect the sterile, open product containers, both empty and filled, from particle contamination. The air velocities required in these barrier systems to achieve the desired number of air changes per hour is significantly less than for a clean room suite, because of the smaller air volume within the barrier. Air qualities of Class 10 or lower are claimed to be easily attainable because of the high air volume turnover rate.

The filling/stoppering line to be used inside a barrier system must be minimal in size and all parts must be easily accessible within "an arm's reach" from the sides of the barrier enclosure. The trend is to design the lines so that they are narrow and run linearly. The type of liquid filler that would potentially minimize the need for setup, adjustment, and trouble shooting is the time-pressure filler. This filling machine employs an accurate pressure control system in the formulated bulk holding vessel which feeds the filling needles and relies on precisely controlled on/off valves on the line running to each filling needle. The valves can be as simple as pinch clamps on the needle tubing, which would necessitate the least amount of preparation and setup for a filling order, because new tubing could be prepared and sterilized for each new order. Another design objective would

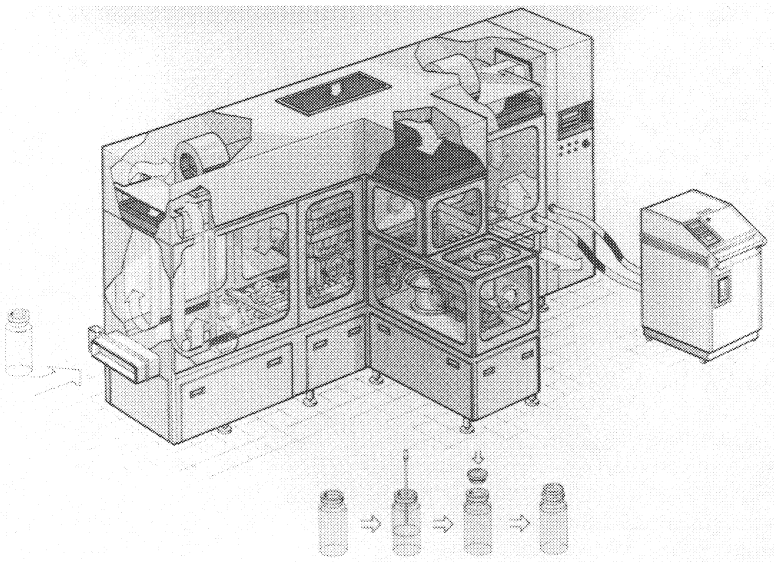


Figure 4. Example of barrier technology system for vial filling (Bosch MLF Machine). Reprinted with permission from TL Systems Corporation.

be to locate as much of the mechanical and electrical hardware for the filling/stoppering machine(s) as possible outside of the barrier system. In this way routine maintenance and repair could be done without having to breach the barrier system.

3. UNIQUE CHALLENGES IN THE ASEPTIC PROCESSING OF PROTEIN PHARMACEUTICALS

3.1. Maintenance of Physical and Chemical Stability during Processing

3.1.1. AVOIDING DENATURATION AND AGGREGATION

Denaturation is defined as the disruption of a proteins's native secondary and tertiary (or quartenary, if applicable) structure and it may occur to varying degrees, depending on the conditions which cause it to

occur. It can be either a reversible or an irreversible event, depending on the extent of the unfolding and the subsequent interactions which may occur between the denatured protein molecules. Such interactions may lead to aggregation and possibly precipitation (Manning *et al.*, 1989).

Denaturation can occur as a result of many types of physical and chemical stresses, for example, interfacial exposures in conjunction with surface tension effects (of which foaming is a special case, because of the additional surface area that is generated), shear stress, freezing and thawing, pH extremes, high temperatures, and high salt concentrations. The following sections will discuss these stresses in greater detail as they affect the outcome of protein drug product manufacturing.

3.1.1.1. Foaming and Interfacial Denaturation

During the processing of protein-containing formulations, denaturation of proteins can occur at air/liquid, liquid/liquid, or solid/liquid interfaces (Lundstrom, 1983). Protein molecules come into contact with these interfaces through diffusion, convective flow, pumping, and agitation.

Assuring complete dissolution and a homogeneous distribution of the formulation components during compounding depends on proper mixing. Depending on the batch size to be manufactured and the tank geometry, the properly sized and designed mixing impeller is selected to achieve the optimal mixing dynamics while minimizing foaming of the solution. Proteins and peptides have varying degrees of surface activity and many formulations of these drugs also contain other surfactants to aid in preventing air/liquid interfacial denaturation. Foaming greatly increases the amount of air/liquid interfacial area in the solution and increases the potential for protein denaturation and insoluble aggregate formation. Even though foaming may be produced by high-shear processes, for example, a high-shear mixer entraining air, interfacial denaturation as a result of foaming is a distinct category of stress that can lead to denaturation in a manner which differs from shear-induced denaturation.

The surface tension of the liquid plus any expansion and contraction at the air/liquid interface is hypothesized to be sufficient to entangle the already conformationally modified proteins adsorbed to the interface, potentially leading to denaturation and aggregation (Kaplan and Fraser, 1953; MacRitchie, 1978). Such an occurrence could adversely affect the potency of the formulation and may also result in the formation of visible particulate matter. Proteins have regions which exhibit greater hydrophobic character than other parts of the molecule. With a protein in an

aqueous solution, the hydrophobic regions will tend to be found in the interior of the molecule, an environment which excludes them from water. Thermodynamically, such an architecture minimizes the protein's free energy. However, this thermodynamic driving force also underlies the interfacial denaturation of a protein as it unfolds to expose or insert its hydrophobic domains into or onto a nonpolar region at an interface. Such locations can be found at the interfaces of the liquid and its surroundings, for example, the gas headspace in the container, at immiscible liquid-like silicone oil droplets on the surfaces of the container or suspended within the liquid, and containment walls, which are made of hydrophobic materials such as polystyrene, polyvinyl alcohol, polyvinyl chloride, or other polymers.

3.1.1.2. Foaming during Compounding and Filtration

Foaming generally occurs as a result of vortexing, which causes air entrainment and bubble formation. Vortexing can be minimized or eliminated by selecting the proper impeller for the application. Impeller diameter and pitch should be considered in relation to the height/width ratio (H/W) of the tank and solution height in the tank. The impeller diameter utilized is generally one-fourth to one-half the tank diameter (Tatterson, 1991, pp. 2–5).

The design of the impeller blades with either a fixed angle (axial flow turbine impeller), a variable, radial pitch angle (such as a square-pitch marine-type impeller), or a radial flow, flat-blade turbine impeller will affect the "pumping" capacity of the impeller and its tendency to cause vortexing (Oldshue, 1997). The variable, radial pitch impeller, termed a "marine impeller," will have greater "pumping" capacity, which will make it suitable only for tanks with relatively large H/W ratios and solution heights $>0.7H$. The fixed-pitch impeller will have less of a tendency to cause vortexing, because there is less "pumping" capacity near the rotor shaft. This phenomenon occurs because the lower angular velocity of the blades near the rotor shaft is not compensated for by an increasing blade pitch, as it is by the marine impeller. This makes the fixed-pitch impeller better suited to tanks with smaller H/W ratios and lower solution heights.

Other procedures that should be considered to optimize tank and impeller setup are properly setting the rotor shaft speed (for reproducible speed control, controllers should have built-in tachometers), positioning the mixing shaft at an angle to and offset from the longitudinal axis of the tank (see Fig. 5), and locating the mixing impeller as close to the tank bottom as practical (Tatterson, 1991, pp. 8, 221). Impellers of varying

designs, examples of which are shown in Fig. 6, are currently available commercially.

Magnetically coupled stirrers such as the MagMixer™ MBI (Lightnin, Rochester, NY) or the NA-mixer® (Nov Aseptic AB, Nödage, Sweden) are alternative types of mixing devices for stainless steel tanks. A shaft, on which the specially designed mixing impeller rotates, must be permanently welded in place on the inside bottom of the tank. On the outside of the tank is mounted the magnetic drive unit which causes the magnetic impeller inside the tank to rotate on the shaft-bearing surface. The impellers are specially designed for use with suspensions or solutions and are sized appropriately for the tank geometry. The advantage of this design is that it generally requires much slower rotational speeds than conventional impellers to achieve optimal mixing. This also results in less foaming.

For smaller batch sizes, <20 L in size, a magnetic stir bar may be utilized in place of an impeller on a shaft. Stir bars must be selected with as much care as impellers to address the same issues as have already been discussed.

Foam can also be formed when two solutions are combined (with one or both containing a surface-active compound) by pouring one into the other directly, or pumping one solution into another through a dip tube that does not extend below the liquid level in the receiving vessel. Such transfers occur when thawed bulk protein, which is stored frozen, must be pooled to yield sufficient material for a drug product batch. The thawed active pharmaceutical ingredient (API) is poured or pumped into a compounding vessel and then formulation buffer is added to dilute the protein to the desired potency (Note: this operation may be done in reverse order; see Section 3.2.1.2 for a discussion of other potential stability issues.) During parenteral production there are other instances of protein-containing solutions being moved from one vessel to another, as when solutions are combined to produce a suspension or to carry out filtration, all of which can yield foam.

3.1.1.3. Foaming during Filling

Foam generated during the filling of a lyophilized formulation can result in the formation of a cake with an unacceptable appearance. This can generally be avoided by utilizing a "bottom-up" or "diving" filling needle movement so that the distance from the bottom of the filling needle to the top of the liquid in the vial is minimized throughout the fill. The flow through a typically sized filling needle used for up to a 10-ml fill

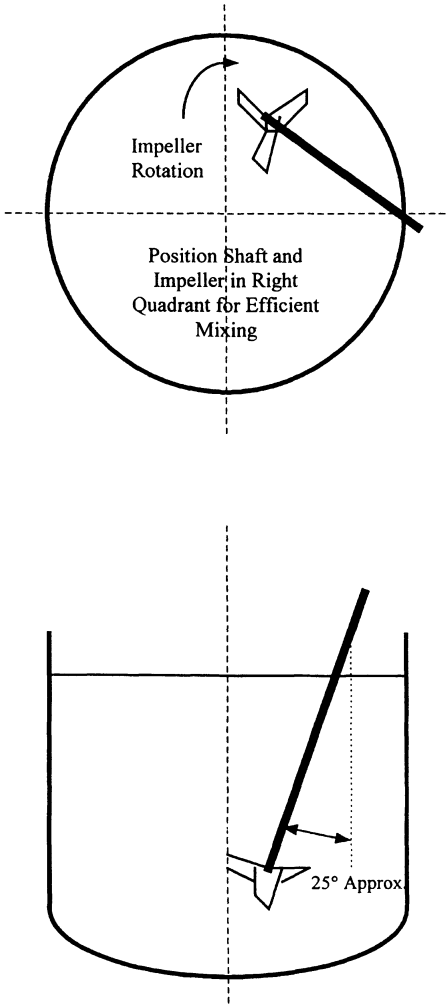


Figure 5. Demonstration of eccentric mixer positioning in a tank to achieve optimal mixing.

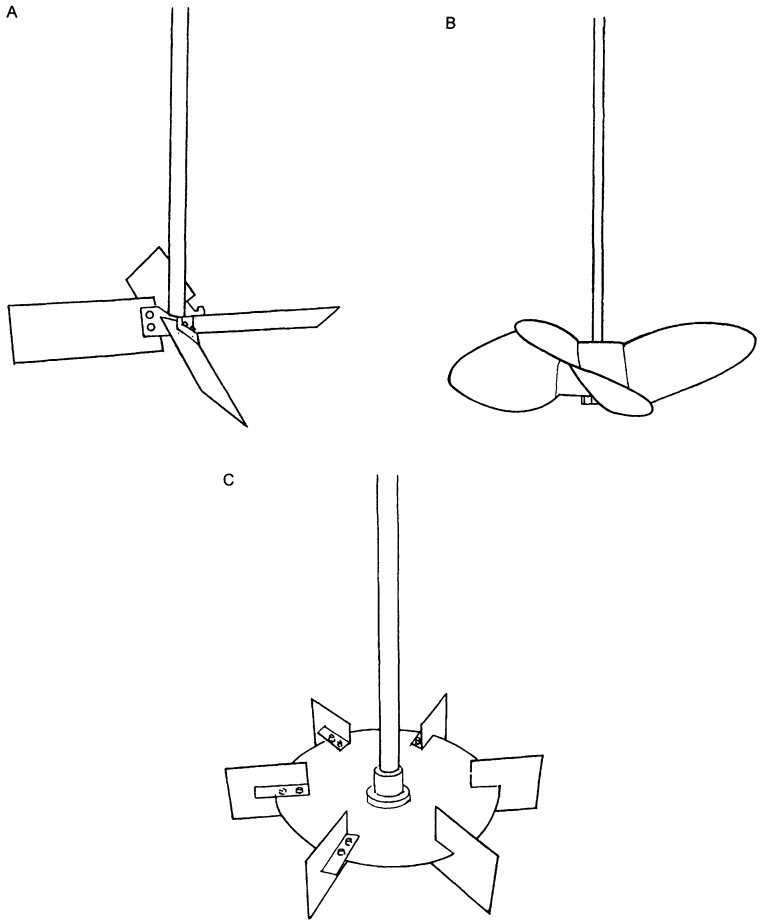


Figure 6. Examples of typical types of mixing impellers: (A) Square-pitch marine type impeller; (B) 45-deg axial flow impeller; (C) Radial flow, flat-blade, disk impeller.

(filling speed of 150–300 vials/minute on line having eight needles) is turbulent in nature [as shown by Reynolds Number calculations (Bird *et al.*, 1960, p. 42); data not shown] and will easily entrain air once it leaves the filling needle. Utilizing larger diameter filling needles and/or slowing the filling rate will also help reduce the formation of foam during the filling operation.

3.1.1.4. Foaming and Comminution of Suspensions

Foaming and difficulty in wetting suspension particles are two problems often encountered when a sterile powder is being combined with a vehicle during compounding (M.J. Akers *et al.*, 1987). The powder must be combined with a minimum volume of vehicle and entrapped air must be removed so that complete wetting can occur. After this has been achieved then the remainder of the vehicle can be added without causing excessive foam formation.

Suspension formulations must be agitated and recirculated during filling in order to maintain a homogeneous distribution of solids. A specialized agitation system must be used for sterile holding tanks which does not compromise the integrity of the tank and the sterility of its contents. Examples of systems that have been used for this application are VIBRO-Mixers and magnetically coupled mixers. As previously described, the stirring rate of a magnetically coupled mixer is generally slow enough so that foam is not generated. On the other hand, a VIBRO-Mixer (B. Braun Biotech International, Melsungen, Germany) can generate foam (see Fig. 7). A VIBRO-Mixer consists of a mixing plate mounted on a shaft that extends into the liquid. The plate is perforated by perfectly round holes, which form the open ends of truncated cones, or venturi openings that have been fabricated into the plate. To mix a solution or suspension the plate is moved vertically in a reciprocating action at high frequency, producing continuous jets of liquid from the venturi openings; the degree of agitation generated is controlled by varying the amplitude of the reciprocating movement. Plate location in the tank, orientation of the venturi openings (forcing flow up or down), amplitude of the plate vibration, and tank bottom design can affect both mixing efficiency and the tendency for foam formation.

A recirculation system employing a peristaltic pump, flexible tubing, and a stainless steel manifold may be used for delivering the suspension formulation to the filling pumps and maintaining its homogeneity. The tubing diameters, the pump, and the manifold dimensions must all be

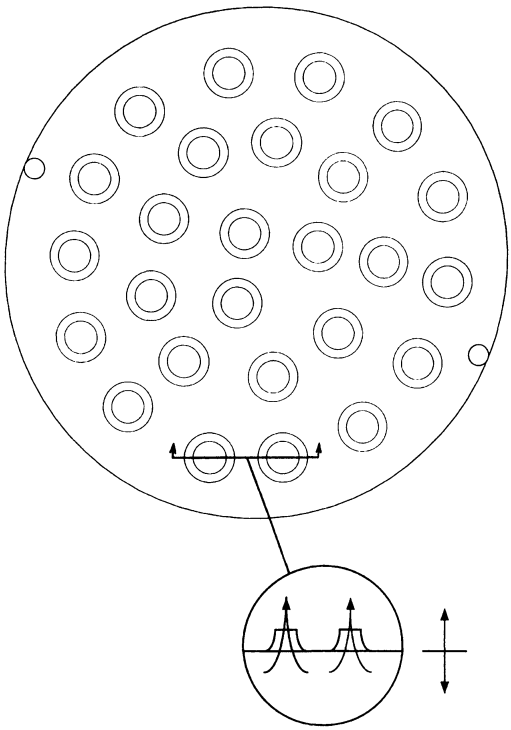


Figure 7. The VIBRO-Mixer stirrer, which consists of a flat plate perforated with hollow truncated cones which are shaped like venturi openings. When the plate is vibrated in a reciprocating manner in a noncompressible fluid, a continuous jet pumping action is created by liquid being accelerated out of the narrow end of the venturi cones.

properly sized so that cavitation and corresponding foaming does not occur at the recirculation rates necessary to maintain suspension homogeneity and adequately supply product to meet the anticipated filling rates.

Another consideration for suspension formulations is that both mixing and recirculation systems must be designed to minimize the comminution of the suspension particles. This is especially critical for crystalline suspensions, because the particles may be fragile, for example, needle-like crystals, and may not be able to withstand a great deal of abrasion or shear stress. Because particle size affects the dissolution rate

of the suspension, a significant size reduction from fracture or abrasion could affect the pharmacokinetic profile of the dosage form.

3.1.2. SHEAR STRESS-INDUCED DENATURATION

Shear stress in fluids is the result of a force being applied parallel to a cross-sectional area of interest, in this case a protein molecule, which produces continuous deformation or flow as layers of the fluid move over each other at different velocities. Such a stress could potentially yield sufficient torque to unfold or denature the molecule. Shear stress may be imparted to a protein in solution during drug product manufacturing from actions such as the flow of the liquid through tubing, flow through a narrow-bore filling needle or pump valve orifice, flow through a sterilizing membrane filter, or the revolving of a mixing impeller or stirrer in a solution.

The author has noted the relative scarcity of literature describing investigations on the shear stress-induced denaturation of proteins, a subject which warrants additional work. However, there are three relevant investigations dealing with the effects of shear stress during flow through tubing and during sterile filtration that are worthy of mention.

Charm and Wong (1970) investigated the effects of shear stress on protein-containing solutions flowing through a tube. The flow velocities of the fluid in the tube are depicted in the schematic shown in Fig. 8. The mean shear rate, dv_z/dr, is defined as the rate of change of the fluid velocity along the longitudinal axis of the tube as a function of the radial position in the tube. Empirically they found that the loss of protein activity is proportional to the mean of the product of the mean shear rate and the time of exposure to the shear, $\langle (dv_z/dr) \times t \rangle$. This product is determined by

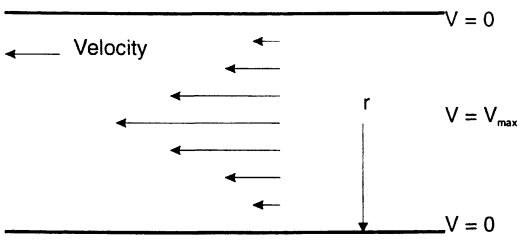


Figure 8. Flow of fluid through a tube.

the tubing length-to-radius ratio (L/r) times the number of passes (N) through the tube:

$$\text{loss of protein activity} \propto (dv_z/dr) \times t = (8/3)(L/r) \times N$$

It follows that at a constant shearing stress the protein activity would decrease with longer exposure times, and for a constant exposure time, greater amounts of shear stress would lead to decreasing protein activities. In addition, the loss of protein activity was found to be additive for intermittent exposures to a shear stress.

For the two proteins investigated, catalase and carboxypeptidase, mean shear rate–time products $>10^5$ were required to cause demonstrable protein activity losses of 5–10%. Higher shear rate–time products resulted in greater activity losses.

Pikal *et al.* (1991) applied this tubing flow approximation to the single passage of protein-containing solutions through a sterilizing-grade membrane filter (pore size = 0.22 μm). Using Charm and Wong's empirically derived formula,

$$\text{loss of protein activity} \propto (8/3)(L/r) \times 1$$

where the radius of a pore $= 0.11 \times 10^{-4}$ cm (0.22 μm filter) and the length of the pore = 0.013 cm, gives

$$(8/3)(0.013\,\text{cm}\ /0.11 \times 10^{-4}\,\text{cm}) = 3.15 \times 10^3$$

The shear rate–time product was approximately 10^3–10^4, suggesting that sterile filtration probably does not produce significant inactivation of proteins because the Charm and Wong data indicated a shear rate–time product of $>10^5$ was required to bring about protein activity loss. They experimentally demonstrated this conclusion by pumping human growth hormone (hGH) solutions through capillary tubes that would approximate the pores of sterilizing-grade filters and showed that no aggregates were produced. Truskey *et al.* (1987) came to a similar conclusion in their studies evaluating the effects of membrane filtration on the conformation of human immunoglobulin G (IgG), bovine insulin, and bovine alkaline phosphatase. The results suggested that any conformational changes which were found to occur were due to the interaction of the protein and the membrane and not to hydrodynamic shear forces.

Impellers or stirrers used during solution compounding or for suspension mixing can also potentially impart significant shear stress to a protein-containing solution. The fluid shear stress is a function of the product of the fluid shear rate and the viscosity of the solution (Oldshue,

1997). The maximum and the average shear rate $\langle (dv_x/dy) \rangle$ around the periphery of an impeller can be calculated if fluid velocity measurements are plotted as a function of the distance from the impeller blade. In practice, these fluid velocities are difficult to measure, so the following relationship gives a general rule of thumb to follow in selecting impeller sizes and speeds for proteins having differing shear sensitivities.

The power P_{app} applied to a system from a turning impeller is proportional to the pumping capacity Q times the velocity work term VW, which is related to the shear rates around the impeller:

$$P_{app} \propto Q \times VW$$

The power P_d drawn by impellers in low viscosity solutions is proportional to N^3D^5, where N is the impeller speed and D is the diameter of the impeller:

$$P_d \propto N^3D^5$$

The pumping capacity of an impeller Q is proportional to ND^3.

By combining the above considerations, one finds that the ratio $(Q/VW)_P$, flow to velocity work (or shear rate) at constant power, turns out to be proportional to $D^{8/3}$. This indicates that larger impellers rotating at slow speeds give greater pumping action and less shear than smaller impellers rotating at higher speeds.

In addition, the shear rate VW of the impeller is proportional to N^2D^2. The relationship of shear rate to impeller diameter result from the fact that the tip of the mixer, where tip speed is greatest, will produce the largest differential flow velocities and thus the largest shear rate. The tip speed of the impeller is defined as (Tatterson, 1991, pp. 2–5)

$$V_{tip} = \pi DN$$

where D is the impeller diameter and N is the rotational speed. Therefore, for different diameters of the same type of impeller operated at identical rotational speeds, the larger impeller will produce a greater shear rate than the smaller one, because of its higher tip speed.

In contrast to the availability of a shear-induced inactivation index empirically derived to estimate protein denaturation during flow through a tube (Charm and Wong, 1970), no empirically derived equation for calculating a shear-rate product for mixing was found in the literature. This would be a very beneficial area of research, which would assist in the appropriate selection of mixers and mixing processes for protein-containing solutions. Research on shear denaturation of a larger variety of proteins would allow more definitive conclusions regarding the importance of shear denaturation of proteins during normal processing.

3.2. Active Pharmaceutical Ingredient (API) Storage and Compounding Issues Affecting Protein Stability

3.2.1. PROTEIN AS API

The physical state of the bulk protein greatly influences the early steps in the drug product manufacturing scheme. The strategies for the storage of the purified proteins or peptides derived from bulk manufacturing processes vary from one manufacturer to another. The decision to store the material as a dried solid, either crystalline or amorphous, or as a frozen concentrate may depend on several factors. Some of these considerations are the relative stability of the protein in the dried versus the solution state, the protein's sensitivity to freezing and thawing, the cost of the facilities and equipment necessary to convert the protein into the solid state (e.g., bulk freeze-dryers, large-scale centrifuges, and drying ovens) and the economics of storing dried powder versus frozen solutions. For storage of bulk material as a frozen solid, the temperature of storage should be considered. Commonly used storage temperatures are $-40°C$ and $-80°C$.

3.2.1.1. Compounding with API Powder

The compounding of a formulated bulk drug solution is the process of dissolving or diluting the proper quantities of the active drug substance, in this case either a protein or a peptide, and the excipients in the appropriate volume of water to achieve the desired concentration or potency. The details of this operation, for example, how the powder components are added, the order of addition of the components, tank and mixer geometry, and mixer design and speed, may all have an effect on the rate of dissolution of the components and the final stability of the active drug substance.

If possible, the dissolution of bulk drug powder should be done at a solution pH that would maximize its solubility and dissolution rate so that the operation could be completed expeditiously without prolonged mixing. Such a dissolution plan may include a pH excursion away from the intended final pH of the formulation into a pH range that would maximize the protein's solubility in order to quickly solubilize the bulk drug. At the completion of dissolution the pH would be adjusted to the final formulation pH. As an example, dissolving zinc insulin crystals during compounding is accomplished by reducing the solution pH to the range of 3.0–3.5 to accelerate dissolution and then raising the pH to neutrality, which is the final formulation pH (Brange, 1987). Such a plan

must be fully investigated during the formulation and process development phases by defining and evaluating the pH–solubility and pH–degradation rate profiles of the protein. Time limits may need to be instituted for such pH excursions during compounding, because the kinetics of a particular degradation mechanism for the protein may be accelerated significantly at the extremes of pH. This is the case for zinc insulin, where the rate of one deamidation reaction is greatly increased in the lower end of the pH range which yields maximal solubility (Fisher and Porter, 1981). In addition, if the pI of the protein is traversed during the pH adjustment, precipitation may occur. This may only be a transient phenomenon; however, it should be simulated in the laboratory before such a pH adjustment is attempted at larger scale.

3.2.1.2. Compounding with Frozen API

If bulk protein is stored frozen, there are several compounding-related issues that should be investigated during process development in order to achieve maximal physical and chemical stability of the protein. These include the method of thawing, the temperature/time relationship for the thaw, and the order of addition of the bulk protein and dilution buffer. Thawing should be carried out in a reproducible manner, in a recirculating water bath, a vibratory shaker bath, an environmental chamber, or under some other controlled condition. Dilution of the thawed bulk protein can be done either by adding the protein to the buffer or vice versa.

Achieving the desired protein potency in the formulated drug product can be accomplished by increasing the protein concentration from infinite dilution (adding API protein to dilution buffer, which mimics the dissolution of dried bulk protein powder in solution) or diluting from the higher bulk protein concentration (adding dilution buffer to pooled API protein). The argument for using the latter procedure is based on the fact that the solution stability of many proteins is enhanced as protein concentration increases (Hanson and Rouan, 1992). In these cases, very dilute protein solutions, such as when API protein is added to dilution buffer, may actually be deleterious.

3.2.2. EXCIPIENTS

Excipients, for example, buffer salts, amino acids, polyols, and preservatives, should be added in an order which would result in a positive

or at least a neutral influence on the active protein, but should not result in their own accelerated degradation. For example, dissolving a sugar such as sucrose in a very low pH solution may be detrimental, because it is much more susceptible to hydrolytic degradation under acidic conditions (Pazur, 1970). Therefore, if possible, such an excipient should be added after the compounding pH has moved closer to neutrality.

The exact form of the excipients that are specified during development of the drug formulation and production processes must be employed in its manufacture for clinical or commercial use. For example, different hydrates of buffer salts may have very different dissolution characteristics. The solubility in cold water of the heptahydrate of dibasic sodium phosphate is quite different from the dodecahydrate, 104 g/100 ml and 4.15 g/100 ml, respectively (Weast, 1971). If the incorrect hydrate is used, these differences may result in incomplete dissolution of the excipients during a set mixing period in compounding. This could result in a pH that is far from that intended for the protein or peptide at that step in the process and could affect either its physical or its chemical stability.

Another example of the importance of using the correct excipients relates to the fact that the solubilities of some proteins are often significantly affected by the ionic strength of the medium in which they are dissolved. Ionic strength is generally most dramatically affected by buffer salts, other salts added to the formulation, and the salt which is generated from the titration with acid or base from the starting pH to its final value. If the compounding instructions call for the addition of one buffer species, say, dibasic sodium phosphate, Na_2HPO_4, and the other species is added instead, say, monobasic sodium phosphate, the salt generated from the additional titrant needed may have an adverse impact on the protein's solubility. Such an error could lead to haze or visible precipitate in the formulation.

3.3. Low Concentration of Protein in Drug Product

Proteins of pharmacological utility, most of which are produced via recombinant DNA or hybridoma technology, are macromolecules which are generally very similar to those found in the human body. These polypeptides, such as insulin, hGH, interleukin-11, interferon γ 1-B, or antihemolytic factor VIII, are all found in low concentrations within the body. The signaling, regulatory, modulatory, or enzymatic activities

through which they exert their effect do not require high physiological concentrations.

Administration of doses on the order of tens of micrograms to a few milligrams are common for these very potent molecules (Facts Comparison Staff, 1996). This would translate into drug product formulations that contain a few micrograms or milligrams of active substance per milliliter. For a protein such as insulin having a molecular weight of approximately 6000 Da, a formulation containing a 3-mg/ml concentration would correspond to 5×10^{-7} *M*. For a protein of molecular weight 60,000 Da in a formulation containing 3 mcg/ml, the molarity would be 5×10^{-11}.

3.3.1. CONSEQUENCES FOR DISPENSING AND COMPOUNDING

For these low-concentration formulations, relatively small amounts of active protein are added during compounding of small clinical batches. Therefore, the dispensing of the protein as active pharmaceutical ingredient must be done accurately on balances having the appropriate level of sensitivity. For example, if the batch record calls for the protein to be weighed to the nearest 0.1 g, then the balance used should have a readout that measures to the nearest 0.01 g.

Dried proteins as active pharmaceutical ingredients may be hygroscopic to varying degrees. This is important to consider during the storage and dispensing operations, because the specific activity of the API may change due to moisture adsorption for those that are hygroscopic. In order to avoid such an occurrence, storage of API should be in air-tight containers and dispensing done in controlled temperature and humidity areas.

During the compounding of a protein formulation the container in which the API is received from dispensing should be thoroughly rinsed after it is emptied into the mixing vessel. For these very potent macromolecules in small batch sizes, the mass of API to be added may not be very great and the amount of drug left as a residue in a dispensing container may be significant. This will help assure that the correct potency is achieved in the formulated drug product. Proper mixing in the compounding tank is necessary before an in-process sample is taken for potency determination (see Sections 3.1.1.2 and 4.3).

One strategy that can be used to help assure that the desired protein potency is achieved during compounding is to add only 70–80% of the theoretically anticipated amount of excipient solution to the mixing tank, add the API, mix, and then perform an in-process potency assay. The results will dictate how much additional excipient solution must be added to reach the

required potency. The success of this strategy depends on the accuracy and precision of the in-process potency assay.

3.3.2. ADSORPTION

Adsorption of small amounts of a protein, already at low concentrations in a formulation, to any of the fill/finish equipment surfaces or the container/closure surfaces could significantly reduce the potency in all or some of the filled vials. The compatibility of a protein and its formulation with the materials used in the fabrication of the equipment employed in the fill/finish process should be investigated during process development and scale-up activities. Adsorption to the components of the container/closure system should be investigated as a part of formulation development and the determination of an appropriate bench-scale manufacturing process.

3.3.2.1. Possible Adsorption Scenarios

Adsorption may be a reversible or an irreversible physical reaction which may lead to conformational and/or chemical modifications to protein molecules. Possible interactions between protein molecules and the surfaces of the equipment in which the manufacturing takes place are depicted below:

$P + S \leftrightarrows P - S$	Reversible (protein conformation unchanged)	(1)
$P + S \longrightarrow P - S$	Irreversible (protein conformation unchanged, but elution from surface does not occur)	(2)
$P + S \longrightarrow P^* - S$ $P^* - S \longrightarrow P^* + S$	Irreversible (protein conformation modified and elution of modified form possible)	(3)
$P + S \longrightarrow P^* - S$	Irreversible (protein conformation modified, but elution of modified form does not occur)	(4)

The reversible reaction shown in (1) may not result in any detectable reduction in protein concentration unless the rate constant for the

dissociation or elution backreaction is significantly lower than the forward association reaction.

Reactions (2) and (4) are probably indistinguishable without a great deal of analytical difficulty (infrared spectroscopy in the reflectance mode may detect the difference); however, they yield a reduction in concentration and/or potency of the protein in the formulation. A finite number of binding sites on the equipment surfaces will eventually lead to their saturation, at which point the adsorption will cease. For a formulation containing a very low protein concentration, on the order of a few micrograms per milliliter, attaining full binding saturation may yield a significant reduction in concentration. This can be addressed by making the number of adsorptive binding sites as few as possible, which can be achieved by minimizing the equipment surface-to-formulation-volume ratio (S/V) for each of the process steps. For example, the sizes of the mixing containers in which the compounding is done should be only slightly larger than the batch size to be manufactured, and the length of any tubing that demonstrates some adsorptive properties should be minimized.

The irreversible reaction (3) is very undesirable, because it reduces the potency of the active compound and generates degradation products. It is possible that this reaction yields inactivated or denatured protein at a liquid/solid interface. Protein inactivation was reported by Edwards and Huber (1992) who studied the adsorption and irreversible inactivation of β-galactosidase at the wall–liquid interface of the containers in which it was stored. The rate of inactivation of dilute solutions of the enzyme was proportional to the wall–liquid surface area and the wall composition (glass or polypropylene). Denaturation of adsorbed proteins may lead to aggregation of the desorbed protein species P* into amorphous or gelatinous particles. Just such a reaction has been seen to occur with the adsorption and aggregation of insulin in implanted pumps and catheters, which led to their blockage (Lougheed *et al.*, 1980).

3.3.2.2. Adsorption to Process Equipment

Equipment adsorption studies can be done in a number of ways. Small quantities of the formulation can be exposed to scaled-down versions of the equipment to be employed in the clinical or commercial manufacturing operation, or small "coupons" of the materials of construction can be immersed in minimal volumes of the formulation. Scaled-down equipment or coupons should be made of exactly the same material and have the same surface finish as the full-scale equipment. For metals such as stainless steel,

the identical alloy (e.g., 316, 316L, 304) with the same surface finish (e.g., #3 finish, electropolished) should be used. Small formulation volumes are used so that even minimal adsorption would be amplified, making it detectable analytically. The exposure time for these studies should be significantly longer than that anticipated for actual manufacturing, so that some safety factor could be built into the results. The temperatures of the exposure studies should cover the range commonly encountered during manufacturing and storage, which could vary from room temperature down to refrigeration temperatures.

Membrane filters, and their metal housings or plastic capsules, used for sterile filtration of the formulated bulk protein solutions should also be tested for adsorptive properties (Brophy and Lambert, 1994; Martin and Manteuffel, 1988; Pitt, 1987). The testing can be done in a dynamic mode where samples of the filter effluent can be collected as a function of time after the beginning of filtration. The samples should be analyzed for the concentrations of both the active protein as well as the excipients. Changes in either could adversely affect the formulation's immediate potency or its long-term stability. Figure 9 shows the results of one such experiment where two types of filtration media (Filter Media B and C) display much greater saturable binding activity than the third (Filter Medium A). The amount of protein bound can be calculated from these curves and its impact on the final protein concentration in the formulated bulk solution can be evaluated.

Measuring the flow decay during the sterile filtration of a formulation is another method for evaluating the potential for adsorptive interactions with filtration media. As seen in Fig. 10, two of the filters (Filter Media A and C) evaluated for use with the protein formulation in question exhibited significant flow decay, whereas the other filter (Filter Medium B) maintained its starting flow rate throughout the time interval of the test. The three filters were made of different polymeric materials and the ones that demonstrated the flow decay may have interacted with the protein in such a way that denaturation and aggregation occurred, leading to physical blockage of the filter. The filter yielding the least flow decay should be the one chosen for the production operations, provided that it is found to be fully compatible with the formulation.

3.3.2.3. Adsorption to Container/Closure Systems

Adsorption of protein to glass vials or stoppers may be addressed in a variety of ways. The glass treatments or alternatives to glass vials described below alleviate the adsorption problem by reducing or eliminating the

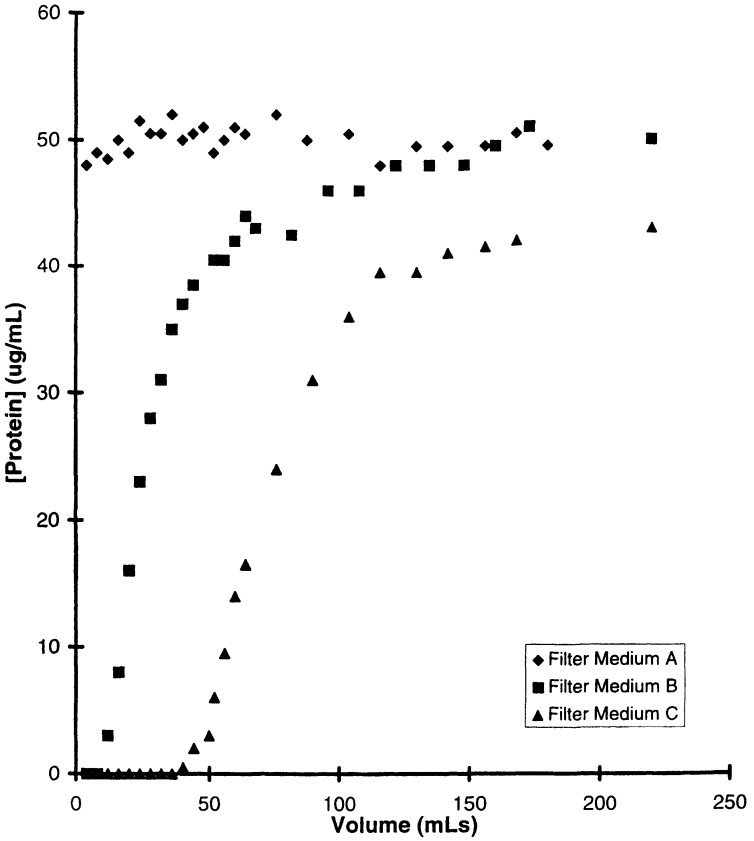


Figure 9. Dynamic binding of a protein to three types of filter media.

electrostatic binding sites on the glass surface. The solutions to such adsorption problems generally have complicating ramifications for the manufacturing process. If a protein adsorbs to a surface via a predominantly hydrophobic mechanism, which, for example, is the case for insulin (Sluzky *et al.*, 1991), many of the alternatives listed here would be counterproductive. These approaches, for the most part, provide a hydrophobic covering or alternative to glass surfaces.

The type of glass routinely used in the manufacture of vials and ampoules for parenteral products is Type I borosilicate glass. This glass is

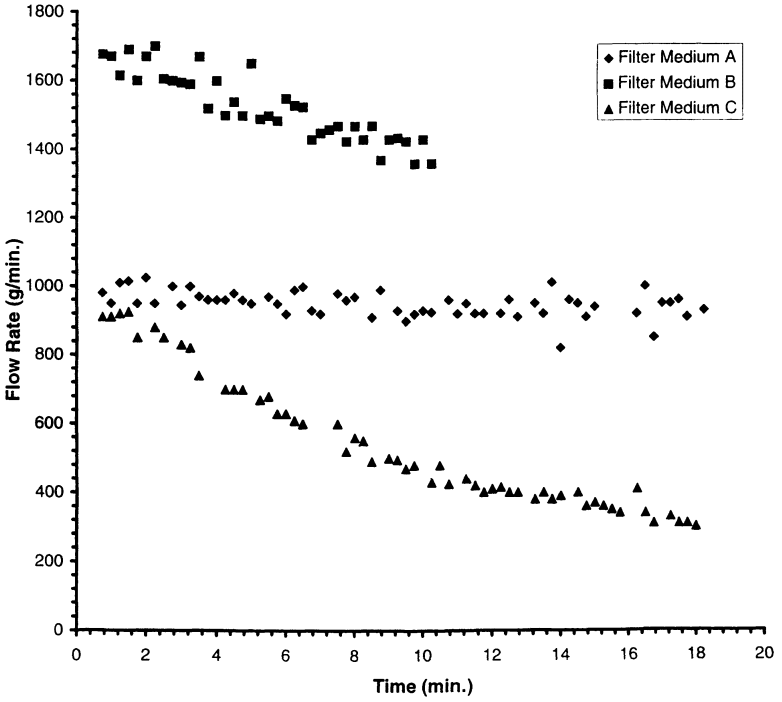


Figure 10. Filtration flow rates for three types of filter media (same media as Fig. 9).

potentially the least reactive and has the least amount of extractables as compared to soda-lime glass (Adams, 1977). Type I borosilicate glass can also be produced with different expansion coefficients as a result of different ingredients and proportions of ingredients being added to the glass formulation. However, regardless of the expansion coefficient of the glass, essentially the same types and numbers of electrostatic binding sites will be present on the surface of the glass, namely the $\equiv Si\text{–}O^- Na^+$ groups.

A "sulfur" treatment is offered by glassware suppliers, which is purported to react with and reduce the number of reactive groups on the glass surface. The treatment involves spraying a dilute solution of ammonium sulfate, $(NH_4)_2SO_4$, into the container before the newly formed container is placed into a lear (oven) to anneal the glass. The high temperature of the lear vaporizes the ammonium sulfate and converts

it into ammonium hydrogen sulfate, which in turn reacts with many of the $\equiv Si–O^-$ groups, yielding a less reactive glass surface (Roseman *et al.*, 1976).

Molded plastic vials fabricated from different polymeric resins are an alternative to glass vials. A polymer called "CZ resin" manufactured by Diakyo of Japan is used in the production of exceptionally clear vials. These vials have been found not to be adsorptive for one recombinant protein which adsorbs to glass with high affinity (unpublished data). One potential drawback in the use of CZ vials, however, is their inability to withstand dry heat oven depyrogenation temperatures. The vials must be depyrogenated during washing and then autoclaved and dried prior to filling. The CZ vials are also significantly lighter than glass vials, which may make their handling difficult on filling equipment designed for glass. In addition, the vials have been shown to be suitable for lyophilization, even though their heat transfer coefficients are less than those of glass (unpublished data). As with all prospective parenteral container candidates, overall compatibility between the protein formulation and the plastic vials must be demonstrated before they could be considered for use.

Siliconization of the glass vial interior is another method for minimizing protein adsorption. Siliconization is a commonly used treatment for syringe barrels to supply lubricity to facilitate plunger stopper movement for the expulsion of the syringe contents. In general, though, the amount of silicone oil required for lubricity is greatly in excess of that needed to prevent protein adsorption. For syringe barrels a 1–2% silicone emulsion dilution (the starting emulsion contains approximately 35% silicone oil plus emulsifying and stabilizing agents, so the oil concentration in the diluted emulsion is 0.35–0.60%, w/v) is sprayed into the barrels after washing. The syringe barrels are then heat-treated at 250–300°C for several hours to bring about "bonding" of the silicone oil to the glass surfaces. Such a high concentration of silicone oil results in significant levels of "free" or unbonded silicone oil. This is undesirable for use with protein-containing formulations, because of the potential for interfacial denaturation of the protein on silicone oil droplets (see Section 3.1.1.1). In fact, a silicone emulsion concentration 1/100th of that used for syringe barrels may be sufficient to prevent protein adsorption in glass vials and not leave excess silicone oil on which denaturation can take place. The exact emulsion concentration to be used must be determined during process development.

Rubber stoppers must generally be siliconized to allow them to be used in automated filling/stoppering equipment and to prevent sticking and clump formation during autoclaving. Their movement in vibratory bowls and along stainless steel tracks as they are moved to the stopper

placement head requires that they have sufficient lubricity. Again, with protein formulations an excess of silicone oil on the stopper can bring about interfacial denaturation and visible particulate formation. Appearance problems linked to excess silicone oil can occur in both solution and lyophilized formulations. Hazy solutions resulting from siliconized stoppers have been linked to the presence of surfactants in the formulation which disperse the silicone oil from the stoppers into small droplets, leading to haze formation (unpublished data). Pavanetto *et al.* (1991) found that the particle levels in parenteral solutions were directly related to the amount of silicone oil applied to the stopper. Groves (1993) stated that the major source of particulate matter in drug products is silicone oil from stoppers, and therefore the amount of silicone oil placed onto stoppers must be minimized. For proteins that are exquisitely sensitive to the presence of silicone oil, Teflon-coated stoppers are available, which should have sufficient lubricity for good machinability and may not require siliconization to prevent sticking during autoclaving. Siliconized or Teflon-coated stoppers should prevent protein adsorption if the adsorption mechanism is electrostatic in nature.

3.4. Microbial Growth-promotive Nature of Protein Formulations

Many protein-containing formulations are manufactured in single-dose configurations where a preservative is not required. These formulations contain not only the active protein, but other excipients, for example, sugars, sugar alcohols, and amino acids, which may make them well-suited to support microbiological growth. This is a critical consideration during the compounding phase of manufacturing and during any hold periods that may occur prior to sterile filtration of the formulated bulk drug product. It is imperative that the bioburden on processing equipment and in raw materials to be used in compounding be as low as possible so that significant microbial growth does not occur during this processing step. Compounding of the formulated bulk may take several hours to complete and, in most cases, it occurs at room temperature. These conditions may allow for the growth of bacteria or fungi in the formulated bulk. This could result in the production of microbial metabolic by-products, such as enzymes, toxins, and endotoxins, which would adversely affect the quality of the drug product.

Effective cleaning procedures for process equipment may reproducibly yield low bioburden levels. However, the greatest assurance of low (zero) bioburdens is obtained by sterilizing the equipment after cleaning. Attention to the bioburden levels in raw materials is also critical. It may be wise to

have raw material specifications call for bioburden testing such that every batch used in production is known to have acceptable bioburden levels.

Multidose products are required to include a preservative in their formulations. Growth of microbial contaminants in the formulated bulk during compounding would be prevented by the presence of the preservative; however, high bioburden levels on equipment or in raw materials should still be avoided.

3.5. Equipment Cleaning

For equipment used in the manufacture of multiple products, cleaning effectiveness is critical for preventing cross-contamination between batches of different products. Avoiding carryover contamination is no less critical if a single-protein product were manufactured using dedicated equipment. The consequences of inadequate cleaning could be that protein material, both native and nonnative, could slough off improperly cleaned surfaces into a succeeding batch of product and adversely affect its quality.

A strategy which avoids the necessity for cleaning reusable equipment during drug product manufacturing is to use as many disposable items as possible. This allows the manufacturer to avoid the cross-contamination or carryover issues for those items that can be discarded after every batch. Tubing and capsule-sterilizing filters are two of the most commonly used disposables.

3.5.1. DEVELOPING A CLEANING PROCEDURE

There are two major considerations when cleaning procedures are being developed for equipment used in the manufacturing of protein formulations. First, if at all possible, protein-containing solutions should not be allowed to dry on equipment surfaces. Second, cleaning solutions and exposure temperatures should be selected to maximize the protein's solubility and not cause denaturation or precipitation of the protein.

Air or evaporative drying of a solution or suspension formulation of a protein on a surface can result in denaturation and aggregation of the protein. In contrast to a protein formulation developed for lyophilization, solution and suspension formulations would not contain additives to protect the physical stability of proteins in the dried state. Evaporative drying can be compared to the changes that occur during the freezing of a

solution. The removal of water results in increases in solute concentrations, which may include high salt concentrations and significant pH changes (Franks, 1990), both of which could result in protein denaturation. The denatured protein mixed with other dried solutes could be in the form of amorphous, crystalline, or gelatinous material. This solid-phase material may have very different solubility in water or other solutions than the native protein or the excipients originally had. Therefore, manufacturing equipment should be cleaned, or at least soaked, as quickly as possible after completion of the processing step during which it was used.

Cleaning, whether using a manual procedure or a CIP system, should follow a well-developed plan for solubilizing and rinsing away both native and nonnative protein molecules as well as excipient solutes. If they can be avoided, detergents should not be used in the process, because it is then necessary to demonstrate that all detergent residuals are also removed from the equipment after the cleaning is completed. In most cases the most difficult solutes to remove from the equipment will be protein. This is especially true if denatured protein has dried onto a surface.

The sequence of cleaning steps, including pHs and temperatures and water rinses, can be planned by examining the pH–solubility profile and the thermal denaturation temperature, T_m of the active protein (Middaugh and Volkin, 1992). If the native protein is soluble in water at the concentration found in the formulation, then the first step in a cleaning procedure should probably be a warm-water rinse to dilute and remove most of solutes on the equipment. The temperature of the rinse should be warm, but no higher than 5–10°C below the T_m of the protein. If the protein has a very low solubility in water, then a water rinse should not be used early in the procedure.

A rinse at the pH of maximum solubility should follow next. In the case of one currently marketed recombinant protein, maximum solubility is at the extremes of the pH range. In this case, a HCl solution in the pH 1–2 range was chosen for the initial rinse after the water rinse. An acidic rather than a basic medium was chosen for this step, because this was the compounding strategy used for initially solubilizing the bulk crystalline powder. A caustic rinse using a NaOH solution followed. Because it was felt to be easier to rinse away acidic rather than caustic residues, another HCl rinse followed. A demineralized water rinse and a water-for-injection rinse ended the cleaning procedure.

For smaller pieces of manufacturing equipment that can fit into a ultrasonic cleaning bath, many or all of the cleaning steps should be carried out there. The ultrasonic bath is effective in solubilizing solutes adhering to equipment surfaces, because of the cavitation generated by the ultrasonic waves in the washing solution.

3.5.2. CLEANING VALIDATION

Cleaning validation must be done on product contact equipment prior to manufacturing the first clinical lots, especially if the equipment is not dedicated to the product being manufactured. The FDA's *Guide to Inspections of Validation of Cleaning Processes* should be consulted prior to planning a cleaning validation exercise (U.S. FDA, 1993b). The validation testing must utilize a meaningful and reproducible sampling procedure, such as evaluating residues in the final rinse water, or swabbing a defined surface area with an appropriate, protein-solubilizing solvent. Development of these procedures should include demonstrating that both native and nonnative proteins can be detected, as well as determining the protein recovery percentage. The advantage of the rinse sampling technique is that a much larger surface area is being sampled than by the swab method. However, the dilution effects from the large volume of the rinse may make any residuals undetectable. Many validation protocols will include both rinse water and swab samples to take advantage of the benefits offered by both.

The strategy for setting acceptance criteria for cleaning validation has historically been based on one of the following considerations:

- *Toxicity or safety*, where the no-effect dose is determined and then is divided by a safety factor to set the limit for a specified surface area
- *Pharmacologically based*, where the lowest clinical dose is established and is then divided by a safety factor (1000 is the most commonly used safety factor)
- *Cleaning process capability-based*, where the lowest cleanable level is established after "heroic" cleaning of the manufacturing equipment

Another important consideration in setting residual specifications is the analytical detection capability. Assay detection limits should be considered in selecting an appropriate analytical method for the cleaning validation exercises; however, the detection limit should not be the sole basis on which a residual limit is established (Jenkins and Vanderwielen, 1994).

The analytical methods selected to quantitate the residual proteins in the samples must have the required sensitivity in order to meet the acceptance criteria. These methods can be either nonspecific [e.g. total organic carbon (TOC), protein-specific dyes or spectrophotometric methods] or product-specific in nature [e.g., enzyme-linked immunosorbent assay (ELISA), reversed-phase high-pressure liquid chromatography (RP-HPLC)]. The ELISA method utilizing monoclonal antibody detector probes is generally so specific that only native protein molecules are detected and not denatured ones. Use of the appropriate panel of polyclonal antibodies as

detector probes can broaden the scope of detection to both native and nonnative protein molecules.

3.6. Adverse Effects from Disinfectants or Sterilants

Aseptic filling areas and lyophilizers (that are not steam-sterilizable) must be disinfected at specified intervals to minimize microbial contamination. In addition, it may be standard practice in some aseptic filling operations to place disinfectants on tubing and sterile fittings prior to making an aseptic connection. For protein formulations, very small amounts of these disinfectants or sterilants may cause chemical modifications to the protein.

Kirsch *et al.* (1993) investigated the potential of a variety of commonly used disinfectants to induce protein degradation. The studies consisted of spiking very low concentrations of alcohols, hydrogen peroxide, phenolics, aldehydes and some combination commercial disinfectants/sterilants into a commercial formulation of hGH. The protein was characterized before and after lyophilization to evaluate the relative effects of the chemical agents in solution and then after the stresses of freezing and drying. Alcohols (methanol and isopropyl alcohol) caused no significant changes in the protein. Hydrogen peroxide caused significant degradation to the monomeric hGH in the lyophilized form, but did not cause aggregate formation (as would be expected, it had no effect on hGH in solution). Formaldehyde and glutaraldehyde both caused massive covalent aggregation. Phenol and other phenolic derivatives caused varying amounts of both reversible and irreversible dimer formation in both the solution and freeze-dried forms. Similar levels of dimer were found with combination commercial products which contained several phenolic derivatives, plus an aldehyde in one case. These results indicate the relative susceptibilities of one particular protein pharmaceutical to residues of chemical disinfectants/sterilants that are commonly used in parenteral drug product manufacturing. Other proteins may exhibit different spectra of susceptibilities to all of the agents described above, with the exception of the aldehydes.

As described in the previous paragraph, aldehydes are very reactive compounds that may chemically modify and crosslink proteins both in solution as well as in a freeze-dried cake (Lubig *et al.*, 1981). Many European pharmaceutical companies have commonly used formaldehyde vapor as a means of sterilizing their aseptic filling areas. Following adequate aeration of the area, the residual levels may be reduced sufficiently so that no adverse effects can be detected in protein products that may be manufactured there. However, sterile holding tanks containing bulk formulated

protein should not be stored in the aseptic areas during such formaldehyde vapor treatment. Most tanks will have air vent filters in place, which may either allow for the ingress of the vapor during the treatment or may adsorb formaldehyde vapor and then slowly release it into the headspace gas in the holding tank. Another potential concern is that some types of tubing used for solution transfer, such as silicone tubing, may absorb the vapor if left in the aseptic area during the gas treatment and then allow the aldehyde to diffuse into any solution that passes through it. Given the sensitivity of proteins to aldehydes, the use of the latter in areas or on equipment used for protein pharmaceutical manufacturing seems risky (Fraenkel-Conrat and Olcott, 1948a,b; Habeeb and Hiramoto, 1968; Saleh *et al.*, 1989).

3.7. Surfactants in Protein Formulations

Many protein formulations contain surfactants in order to stabilize the protein conformation and prevent interfacial denaturation during dosage form manufacturing, shipping, storage, and end-user manipulations (Levine *et al.*, 1991; Manning *et al.*, 1989). The mechanism of this protein-stabilizing characteristic has variously been described as being due to the exclusion of protein molecules from interfaces by the surfactant molecules and/or the binding of surfactant molecules to the protein, thereby enhancing the stability of the protein's native state (Wang and Hanson, 1988).

Surfactants are surface-active compounds, that is, they readily adsorb to the air–liquid interface in a solution. This phenomenon occurs because surfactant molecules are linear, having distinct hydrophobic and hydrophilic ends. They may align at an air/liquid interface with the hydrophobic end extending into the air and the hydrophilic end in the solution. The hydrophobic end is mainly a chain of methylene groups of varying lengths, and the hydrophilic end can be either ionic or nonionic in nature. Nonionic surfactants interact with protein molecules via hydrogen bonding, which is pH-independent, whereas the electrostatic interactions between ionic surfactants and proteins arise from oppositely charged moieties on the respective molecules. The ionization state of ionizable groups, especially on the protein molecules, is pH-dependent, whereas ionic groups on the surfactant molecules, for example, sulfate or sulfonate sodium salts are, within the scope of this discussion, pH-independent.

Surfactants reduce the surface tension of a solution, thereby increasing its "wetting potential." This characteristic, as well as their

ability to interact with hydrophobic molecules to solubilize or disperse them in solution, greatly enhances a solution's "extracting potential" from container/closures or process equipment used in drug product manufacturing. The presence of surfactants in a protein formulation may require a more rigorous approach to the optimization of stopper cleaning and siliconization and the selection and cleaning of other polymeric or elastomeric equipment, such as tubing and gaskets. Surfactants may be a difficult excipient to remove from multiuse product contact equipment during cleaning operations, because of the tenacity with which they may adsorb to a variety of equipment surfaces.

As previously described, silicone is placed on virtually all stoppers to allow for machinability and prevent sticking during autoclave sterilization (see Sections 2.1.3.2 and 3.3). Silicone oil from stoppers (or from siliconized vials or syringes/cartridges) can be dispersed by a surfactant in a formulation. This dispersion, depending on the amount of silicone oil extracted, can generate a hazy or opalescent appearance due to light scattering. As a rule of thumb, the amount of silicone oil applied to stoppers used for protein formulations should be minimized for appearance reasons as well as to avoid the potential for interfacial denaturation of the protein.

Protein denaturation and aggregation is postulated to occur at the interface between two immiscible liquids (Sluzky *et al.*, 1992), such as an aqueous solution and dispersed silicone oil droplets. In a case where there is an excessive amount of silicone oil dispersed into a surfactant-containing solution, the surfactant may actually exacerbate the potential denaturation problem, because by dispersing the silicone into smaller droplets it is actually increasing the amount of available surface area on which denaturation can occur. The polymeric molecules of silicone oil, with which the hydrophobic ends of the surfactant molecules interact, may also reduce the effectiveness of the surfactant molecules in excluding protein molecules from a liquid/liquid interface as compared to an air/liquid interface. This may occur due to a reduction in the packing efficiency of surfactants at the silicone oil interface in comparison to that at an air interface.

4. DEVELOPING ASEPTIC PROCESSES FOR DRUG PRODUCT MANUFACTURING

In the overall scheme of drug product development, manufacturing process development should play an increasingly greater role as a drug product candidate moves from the production of informal stability lots to clinical lots and finally to commercial batches. It is likely that the drug product formulation, and perhaps even the dosage form, may change (e.g.,

lyophilized to liquid formulation) as clinical trials progress. Such a scenario would suggest that minimal process development effort be expended to understand and optimize manufacturing parameters during Phase I and II trials. Once the product is defined for Phase III pivotal trials, then an extensive effort will be required to lay out the production process in detail, scale it up, define the critical process parameters, and then determine their optimal target values and allowable ranges. All of these efforts should culminate in a validated commercial drug product manufacturing process.

4.1. Bench-Scale and Early Clinical Trial Manufacturing Process Development

Responsibility for the development of the bench-scale manufacturing process lies with the formulation development and process development scientists. Definition of the bench-scale process depends on the following considerations:

- Compatibilities of the formulation with the materials of construction of potential process equipment
- Selection and preparation of the container/closure components
- Type of sterilizing filter selected
- Any sensitivities of the protein observed during preformulation or early formulation development work that must be accommodated in the bench-scale process (e.g., extreme shear sensitivity requiring greater attention to mixing, pumping, and filling parameters)
- If applicable, a lyophilization cycle developed in a laboratory-scale freeze dryer

The bench-scale process devised with these items in mind should be suitable for manufacturing informal stability lots, tox material, and other preclinical supplies.

Most of the technical details of the bench-scale process, with the exception of equipment sizes and the lyophilization cycle parameters, may be transferred to a cGMP pilot or commercial production facility for the manufacturing of cGMP drug product for Phase I, Phase II, and early Phase III clinical trials. Equipment should be selected to match as closely as possible the clinical batch sizes to be manufactured. Before transferring the lyophilization cycle to a larger facility it would be prudent to run the bench-scale cycle in a freeze-dryer approaching in scale the one to be used for the clinical batch, and, if possible, in the actual freeze-dryer to be used

for the clinical fill/finish operation. The primary objective would be to verify the sufficiency of the primary and secondary drying times which had been previously defined in a smaller freeze-dryer. Such a technical qualification could be done with vials filled with formulation buffer alone or as a "seeded buffer" load, where some vials containing active protein are seeded into a load of vials containing only formulation buffer. The "seeded" batch approach would conserve valuable API, but would allow for an evaluation of residual moisture level variability in a full-scale load as well as a limited evaluation of the lyophilization effects on protein molecular integrity.

4.2. Phase III Clinical and Commercial Manufacturing Process Development

Once the decision has been made to move a product and a dosage form to market, the role of a process development group and a pilot development facility increases significantly. At this point, a plan should be devised to optimize and scale up the bench-scale process to a commercial manufacturing operation. The technical details of a bench-scale process would consist of, at the very least, the container/closure system, a sterilizing filter type, and, if the product is to be lyophilized, an unoptimized cycle. The following general process development scheme might be a first step in assembling a more detailed plan:

Bench Scale

1. Optimize the process at bench scale
2. Determine the compatibilities of potential materials of construction with the formulation
3. Define the protein's and formulation's sensitivity to potential processing stresses
4. Determine critical process parameters (CPP) at bench scale using a design-of-experiments (DOE; Torbeck and Browning, 1996) approach where the effects of multiple variables are evaluated in a matrix*
5. Define the allowable CPP ranges at bench scale

*An article by Li *et al.* (1993) provides an example of the use of DOE to investigate a process development problem. The study reports on the use of an eight-run Plackett–Burman design matrix to determine the critical parameters affecting particle contamination from siliconized stoppers.

Pilot Scale

6. Test validity of bench-scale CPP at pilot scale
7. Evaluate all process steps for scale-up problems
8. Define allowable hold times and temperatures during and between process steps
9. Define and validate the unit operations for component cleaning and sterilization
10. Define the cleaning, preparation, and sterilization of product contact equipment

Commercial Scale

11. Perform technical qualification fills at commercial scale
    a. Evaluate all process steps for scale-up problems
    b. Verify CPP and ranges from pilot scale

4.3. Prevalidation Process Development Studies

The following prevalidation studies for each process step are examples of a more detailed version of the plan laid out above. These studies should have the objective not only of defining the CPP and their allowable ranges but also of building flexibility into the manufacturing process. This is not meant to be an exhaustive list but suggests representative topics for consideration during planning for a solution or lyophilized product.

1. Active pharmaceutical ingredient
   a. Establish stability profile
      i. For frozen protein API: effects of multiple freeze-thaws, thawing temperatures and times, hold times at different temperatures, other handling stresses
      ii. For dried powder API: effects of storage temperature and time, humidity levels (water vapor adsorption isotherm), storage method after dispensing
   b. Define compatibility with equipment and processing variable ranges
2. Formulation excipients
   a. Define optimal storage temperature and humidity ranges and expiry time or retest frequency
   b. Define optimal dispensing procedure and storage afterward
3. Rubber closures (assuming compatibility of primary closure with formulation has been established)
   a. Establish storage temperature range before processing
   b. Establish expiration dating prior to processing

c. Establish closure preparation procedure
   i. Define wash cycle (equipment, time, temperature, use of detergents, rinse times)
   ii. Determine minimum silicone levels
   iii. Define optimal siliconization process
   iv. Define time/temperature of autoclave sterilization cycle
   v. For lyophilization stoppers, define optimal drying time/temperature after autoclaving to minimize absorbed moisture in the closures
d. In conjunction with formulation scientist, identify secondary closure; carry out steps a–c

4. Compounding of formulation buffer (for diluting frozen protein API)
   a. Define order of addition of excipients
   b. Optimize mixing vessel (size and height/width ratio) and mixer configuration (type, impeller, mixing speed range)
   c. Define minimum and maximum volume
   d. Define required mixing time and temperature
   e. Define allowable hold time of completed buffer
5. Compounding of bulk drug product
   a. For frozen API + formulation buffer
      i. Define order and manner of addition of each solution
      ii. Determine effects of shear stress and agitation on protein
      iii. Optimize mixing vessel (jacketed tank required?) and mixer configuration
      iv. Define maximal hold times and temperature range with and without a filtration step to control bioburden.
   b. For dried powder API
      i. Define order and manner of addition of API and excipients
      ii. Determine effects of shear stress and agitation on protein
      iii. Optimize mixing vessel and mixer configuration
      iv. Define maximal hold times and temperature range with and without filtration step to control bioburden
6. Sterile filtration (assuming compatibility of primary filter with formulation has been established)
   a. Perform bacterial retention test
   b. Confirm bubble point
   c. Establish compatibility with scaled-up filtration apparatus
   d. Determine maximum flow rate
   e. Assess effects of filtration on protein
   f. Establish maximum time for filtration to occur and evaluate effects on formulation

g. Define maximal hold times and temperatures after filtration
h. In conjunction with formulation scientist, identify secondary filter; carry out steps a–g

7. Aseptic filling
   a. Determine effects of filling speed on protein; optimize pump and needle size
   b. Establish maximum filling rate
   c. Determine formulation compatibility with materials for construction of pumps
   d. Establish maximum filling time and temperature
   e. Define maximum hold times and temperature for interrupted fill
8. Lyophilization (if applicable)
   a. Optimize the bench-scale lyophilization cycle to make it more economical and robust
   b. Define the targets and allowable ranges for all cycle parameters
   c. Evaluate cycle parameters for scale problems
   d. Define minimum/maximum number of vials per load
9. Capping
   a. Evaluate seal integrity of capped vials from commercial process [several types of tests for seal integrity have been described by Hofmann (1993, pp. 139–141), including dye leak testing, microbial media fills, microbial broth challenges, and residual seal force testing]
10. Cleaning of product contact equipment
    a. Define the limit for residual protein levels after cleaning
    b. Design sampling method and analytical testing for protein residuals
    c. Define cleaning agents and order of use
    d. Evaluate cleaning procedure for excipient removal and modify procedure if required
    e. Validate the cleaning method, the sampling method, and the analytical method

The results from these prevalidation studies would form the basis and the rationale for much of the sampling and testing plan embodied in the Drug Product Process Validation Protocol.

5. CONCEPTUAL FRAMEWORK FOR DRUG PRODUCT PROCESS VALIDATION

The validation of drug product manufacturing is a FDA requirement for licensure of all products and is described as "establishing documented

evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes" (U.S. FDA, 1987, p. 2). Such an exercise also has an economic benefit for the pharmaceutical company, because it generally results in a better understood and controlled manufacturing process, which should translate into fewer product losses from poorly developed processes. The Validation Master Plan is the key planning document and is assembled to define the overall strategy to be followed in preparing for, writing, and then executing and evaluating the Product Validation Protocol, and also for carrying out or reviewing the adequacy of facility, utility, and equipment qualifications and process validations, for example, lyophilizer validation, filling line validation, and autoclave sterilization validation.

5.1. Validation Master Plan

The objective of the Validation Master Plan is to define a comprehensive approach for demonstrating and documenting that the overall drug product manufacturing process is reproducible and results in drug product that will consistently meet predetermined release and stability specifications. The strategy laid out in the Master Plan, which is designed to lead to the completion of a successful validation effort, encompasses facility, utility, and equipment qualifications, formulation and process development experimentation, specific unit operation qualifications and validations, the writing and execution of a Product Validation Protocol, and the planning and production of sufficient numbers of drug product batches at the appropriate scale to demonstrate stability and product consistency.

The Master Plan for a prospective validation effort should include at least the following items:

1. A brief description of the product dosage form and how it will be used
2. A brief process outline
3. Definition of responsibility for writing, reviewing, approving and monitoring the Master Plan
4. Definition of the procedure (reviewing and approving) for amending the Plan and Protocol
5. Definition of responsibility for reviewing plans and results of formulation and process development prevalidation experiments
6. Definition of responsibility for planning and coordinating the manufacture of stability, product consistency, and product validation lots

7. Definition or responsibility for writing, reviewing, approving, and executing the product validation protocol
8. Definition of responsibility for collecting the product validation data, evaluating them, and writing the Final Report
9. Definition of responsibility for reviewing and approving the Final Report
10. Definition of the components minimally included in the Validation Protocol
11. Definition of the components minimally included in the Final Report
12. Postapproval strategy for revalidation of the manufacturing process
13. Definition of responsibility for assuring the completion or adequacy of facilities, utilities and equipment qualifications, and process validations

5.2. Fill/Finish Plan for Generating Stability Lots and Product/Process Consistency Lots

Regulatory requirements call for the production and stability testing of three representative product batches at either pilot or commercial scale in order to establish expiry dating at product approval. Preliminary data from these three lots will be included in the regulatory filing for licensure and will be continuously updated through and after approval. It is generally accepted that 12 months of data should be included in the submission; therefore, manufacturing of the stability lots must commence approximately 14 months before filing so that the testing can be completed and the results included in the regulatory document.

Consistency lots are required for the regulatory submission to supply the FDA with evidence that a company has sufficient manufacturing experience with the product to assure that it can be produced reliably. These data, process as well as release testing, are included in the submission as a part of the representative batch history section. According to the latest guidelines, these product batches may be pilot scale, provided the process is representative of the commercial-scale process.

5.3. Product Validation Protocol

Product validation is designed to demonstrate through an extensive sampling and testing plan, over and above release sample testing, that the

manufacturing process is "under control." In statistical process control (SPC) parlance, being under control means that the inherent variability of each unit operation is defined and that the process output consistently remains within this boundary of expected results.

In order to demonstrate this process control, the Product Validation Protocol should be executed for three lots of product, ideally at the maximum commercial batch size. If possible, multiple lots of components and raw materials should be utilized to further demonstrate the robustness of the manufacturing process. In addition, if more than one piece of major equipment (e.g., lyophilizers, filling lines) are intended for use in commercial manufacturing, then each of them should be included in the validation exercises.

The Product validation Protocol should minimally contain the following items:

1. Process flow diagram
2. Description of the equipment employed for the manufacturing process
3. Rationale for the design of the Validation Sampling and Testing Plan based on summaries of the prevalidation work done by formulation and process development groups (see Section 4.3)
4. Validation sampling plan
   a. Instructions for numbers and types of samples to be taken
   b. Types of analytical assays to be run on each sample
   c. Statistical analysis methods to evaluate the data
5. Acceptance criteria

Validation will be considered successful if the process yields drug product that meets release specifications, all validation sample testing meets acceptance criteria, and the lots are dispositioned as releasable for three consecutive lots.

5.4. Product Validation Final Report

After the successful execution of the Protocol on three consecutive production lots, the data are assembled, analyzed, and evaluated and a Final Report is written. This report is made available to the FDA for their review, but is generally not included in the regulatory submission. Depending on the FDA agency involved in reviewing the submission, it may be necessary to have the Final Report completed for the preapproval inspection, but in all cases it must be completed by the time of product approval and launch.

The Final Report should minimally include the following components:

1. Product Validation Protocol
2. Manufacturing batch records for the three lots
3. Release sampling testing plan and results
4. All deficiency reports and reports of investigation that would explain their disposition
5. Disposition of the lots
6. The raw analytical data for the validation tests
7. A statistical analysis of the validation data
8. An evaluation of the release and validation results for the three lots
9. Conclusions

ACKNOWLEDGMENTS

The author wishes to recognize the thought-provoking conversations that helped formulate the considerations and the development/validation approaches described in this chapter. Among the individuals involved were Michael Boenitz, Donald Sipe, Fen Fang Bond, Jennifer Juneau, Christopher Riel, Richard Northey, Dr. Thomas Spitznagel, Dr. Lawrence Bush, Dr. Kasra Kasraian, Dr. Nicholas Warne, and Dr. Godfrey Amphlett.

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