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MICROBIAL AND VIRAL FILTRATION

Several physicochemical methods exist to ensure the safety of biological and biopharmaceutical products in manufacture. Whereas physical methods such as heat and radiation may be used, these are often not viable options owing to detrimental effects on product quality. For example, in the case of products that are heat labile or biochemically complex, these methods may result in alteration of the chemistry or function of the product, or random adsorption of key components. Filtration (qv) is the separation of particles from a fluid (liquid or gas) by passage of that fluid through a permeable medium. Sterile filtration ensures complete removal of viable organisms. Advances in membrane technology (qv) have resulted in the availability of filtrative devices for the removal of viruses in addition to bacteria. Thus membrane filtration is becoming increasingly the method of choice for sterilization of biologicals, especially when the product is heat labile, because the filtration process is inherently nondestructive.

An overview of the general principles of filtration having specific application to bacterial and viral removal is given herein. The emphasis is on ensuring that the sterility and/or safety of biologicals and biopharmaceuticals be maintained.

1. Filtration for Bacterial Removal

The introduction of parenteral drugs at the beginning of the twentieth century created a concomitant need for a suitable method to ensure adequate sterilization of these biochemically complex heat-labile products. Several different types of filters were introduced into the industrial arena: porcelain filter cartridges, asbestos– cellulose layers, and membrane filters. The porcelain filters (Chamberlain) were used extensively for the manufacture of antibiotics. Problems associated with cleaning and concerns over cross-contamination of products arose, however, and these filters fell into disuse. The first filter medium to be used on an industrial scale was the Seitz EK Filter (EK = *entkeimung* or germ removal). The asbestos content of that filter limited its applications. The first membrane disks were introduced in 1929 and represented a breakthrough in filtration technology. Four decades later membrane filter cartridges were introduced for processing large batches of parenterals.

The earliest commercially available filters were manufactured in two pore sizes: 0.45 and 0.8 μm. The 0.45 μm-rated membranes were considered to be sterilizing-grade filters and were successfully used in the sterile filtration of pharmaceuticals and parenterals. The membrane filters were qualified using *Serratia marcescens*, a standard bacterium, having dimensions of 0.6×1 μm. However, in the late 1960s it became apparent that the matrix of the 0.45 μm-rated filters could be penetrated by some pseudomonad-like organisms (1). For sterile filtration applications in the 1990s, 0.2 μm-rated membranes are the industry standard in the manufacture of sterile parenterals and pharmaceuticals.

2 MICROBIAL AND VIRAL FILTRATION

1.1. General Principles

1.1.1. Mechanisms of Filter Retention

In general, filtrative processes operate via three mechanisms: inertial impaction, diffusional interception, and direct interception (2). Whereas these mechanisms operate concomitantly, the relative importance and role of each may vary.

Direct interception refers to a sieve-type mechanism in which contaminants larger than the filter pore size are directly trapped by the filter. This sieve retention mechanism of particle arrest is the mechanism of choice and occurs owing to geometric or spatial restraint. This type of particle arrest is considered to be absolute, that is, it is independent of filtration conditions.

Inertial impaction involves the removal of contaminants smaller than the pore size. Particles are impacted on the filter through inertia. In practice, because the differential densities of the particles and the fluids are very small, inertial impaction plays a relatively small role in liquid filtration, but can play a major role in gas filtration.

Diffusional interception or Brownian motion, ie, the movement of particles resulting from molecular collisions, increases the probability of particles impacting the filter surface. Diffusional interception also plays a minor role in liquid filtration. The nature of liquid flow is to reduce lateral movement of particles away from the fluid flow lines.

1.1.2. Types of Filters

In general, there are two types of filters used for microbial removal: depth and membrane. The first type removes microorganisms and particles mainly through retention by entrapment or impaction and adherence. These rely on filter matrix depth to achieve particulate contaminant retention. However, using depth filters, microbial cells may sometimes be set free because of high differential pressure, the high flux of fluid passing through the filter, or filter matrix shifting. The primary mechanism of bacterial cell retention by membrane filters is the sieving effect, due to the highly stable, uniform pore matrix, so that trapped cells are not released. Thus membrane filtration is the method of choice for pharmaceutical and biological applications where absolute microbe retention is required. Membrane filters used for sterile filtration applications are typically constructed from, but not limited to, polymers such as cellulose esters, nylon, polyesters, polytetrafluoroethylene (PTFE), poly(vinylidene fluoride) (PVDF), polycarbonate, polypropylene, and polysulfone.

1.1.3. Membrane Filter Ratings

Filters are rated based on the ability to remove particles of a specific size from a fluid. There is, however, no standard on which method is to be used to specify performance. In general, the absolute rating, or cutoff point of a filter refers to the diameter of the largest particle, normally expressed in micrometers which can pass through the filter. The absolute rating is determined under carefully controlled conditions using industry-accepted reference standards, such as silica suspensions, latex beads, or microorganisms. In a filtration system, the actual form of the contaminants is not necessarily spherical, in which case the nominal diameter is generally taken as the largest of the linear dimensions. Many filter manufacturers use a nominal filter rating, which is an arbitrary value determined by the filter manufacturer and expressed in terms of percentage retention of a specific test contaminant (usually latex or glass beads) of a given size distribution. This nominal rating also represents a nominal efficiency figure ($\sim 50 - 95\%$), or more correctly a degree of filtration. Nominal rating standards are, however, arbitrary and thus comparison of nominally rated filters is imprecise. This rating system is not used to characterize sterilizing-grade filters.

Sterilizing-grade filters require that biological retention capability be evaluated using a microbial challenge test. *Brevundimonas (Pseudomonas) diminuta* (ATCC 19146) is the standard test microorganism used for the validation of sterilizing-grade filters. By U.S. FDA definition, sterilizing-grade filters refer to filters

which can remove a minimum concentration of 10^7 colony forming units per square centimeter (cfu/cm^2) of *Brevundimonas (Pseudomonas) diminuta* (ATTC 19146) and yield sterile effluent (3).

1.1.4. Filter Selection

A variety of product- and process-related factors govern filter selection. Considerations include the characteristics of the fluid to be filtered, ie, its chemical composition and compatibility with the filtration system (inclusive of the membrane, filter hardware, piping, etc), the level of bioburden present, specifications on effluent quality, the volume of product to be filtered, flow rate, and temperature.

1.1.4.1. Membrane-Feed Compatibility. The feed stream must be compatible with the membrane selected. The composition of the feed as well as pH and operating temperature must be considered. Materials having excessively low or high pH may not be compatible with certain membrane polymers. The temperature of operation must fall within the membrane manufacturer's recommended temperature range. Most fluids that are sterile filtered are water-based and thus compatible with most membrane materials, such as polyamides, PVDF, polysulfone, and cellulose acetate. In addition to the compatibility of the membrane itself, the compatibility of the filter components, such as the cage, core, and supporting materials, must also be considered. For sterile processes, the biological safety of the membrane filter or filter cartridge must be demonstrated by the performance of the USP ⟨88⟩ Class VI (121 C) Plastics Test for Biological Reactivity. Because there is no specific listing for materials in contact with pharmaceutical products, filter materials of construction are often selected based on the listing for food contact in the *Code of Federal Regulations* (4).

1.1.4.2. Effluent Quality. The criteria to be met by the effluent or filtrate must be clearly defined. For aseptic processes a typical requirement is sterilization through an 0.2 μm-rated sterilizing-grade filter, as defined by ASTM Standard F838-83 (5). The objective of the filtration is to remove contaminants from the process feed without compromising product integrity. Consequently, the filter system must not add anything significant, eg, filter materials of construction or constituents extracted therefrom, to the process fluid, neither should a desired component be removed from the fluid being filtered. Sources that could possibly contribute contaminants to the process fluid include the piping and connections in a process, the filter or associated components such as the cage, core, and supporting materials, or the membrane itself. In some instances, there may be what is referred to as migration of media, ie, dislodging and sloughing of the membrane or support material. Many filter manufacturers document the extractables level of a particular filter in an appropriate solvent in terms of a nonvolatile residue (NVR). These extractables are typically composed of the oligomers of materials present in the filter element or additives. Filter manufacturers address the issue of effluent quality requirements by the performance of appropriate tests on filter samples from manufacturing lots. These tests include cleanliness: per USP limits under Particulate Matter in Injections ⟨788⟩ and conformance with requirements for a nonfiber-releasing filter (6, 7); oxidizable substances: per USP limits under Purified Water after Flushing; pH: per USP limits under Purified Water after Flushing; and pyrogens: per USP limits under Bacterial Endotoxins Test as determined using the Limulus amebocyte lysate (LAL) reagent with an aliquot from a soak solution.

1.1.4.3. Flow Rate Requirements. Flow rate, measured in units of volume per unit time, is dependent on pressure, P, and resistance, R. The flow rate achievable through a filtration system is directly related to the applied differential pressure and inversely related to the resistance to flow. Viscosity of a fluid has a direct effect on resistance. Fluids having higher viscosities are more resistant to flow and the resultant pressure drop across the filter is greater. Consequently, a greater applied pressure is required to maintain the process flow rate. The smaller the pore size rating of a filter, for a fluid of a given viscosity, the greater the resistance to flow. All other factors being equal, if the pressure on a fluid is increased, then the flow rate of that fluid increases. However, if the resistance to flow is increased, such as by membrane plugging, then flow decreases. Additionally, an initial high pressure (and flow rate) can lead to premature plugging, especially for products that may contain gels, as, for example, biological products.

1.1.4.4. Pressure Considerations. All components of a system contribute a resistance to flow which results in pressure drop. Pressure drops or losses in a system can be caused by piping, connections, valves, and filling heads, as well as by the filter and its assembly. The pressure drop through a clean filter assembly results from the sum of component pressure drops, including filter housing, filter hardware, and filter membrane. As filtration progresses and particulates clog the filter, pressure drop increases. In choosing a filter, therefore, it is necessary to provide an adequate pressure source, not only to overcome the resistance of the filter, but also to permit flow to continue at an acceptable rate as the medium plugs. This allows full use of the effective dirt-holding capacity of the filter. If the ratio of the initial clean pressure drop through the filter to total available pressure is disproportionately high, unacceptable flow quickly results even though the capacity of the membrane for collecting dirt has not been exhausted.

1.1.4.5. Temperature Requirements of a Process. The temperature of filtration may affect the viscosity of the fluid, the corrosion rate of the housing, and filter medium compatibility. Elevated temperatures tend to accelerate corrosion and may weaken the gaskets and seals of filter housings. In general, the viscosity of fluids decreases with increasing temperature. Filtration of highly viscous fluids may be conducted at elevated temperature (8). For example, pharmaceutical products containing oil or a lipid emulsion as a drug carrier may require filtration at elevated temperature to enhance filterability characteristics.

1.2. Sterile Filtration of Liquids

The only true test of a sterilizing-grade filter is its microbial retention capability. By FDA definition, sterilizing-grade 0.2 μm-rated filters refer to filters which can remove more than 10^7 cfu/cm^2 of *B. diminuta* and yield sterile effluent. The challenge method for conducting a liquid bacterial challenge is detailed by ASTM F838-83 (5). A schematic of a test setup is presented in Figure 1. The bacterial challenge, *B. diminuta* at a minimum concentration of 1×10^7/cm^2 of filter area, is passed through the filter suspended in a sterile carrier fluid under standard test conditions. Bacterial concentrations are determined in the input as well as in the effluent. Standard microbiological methods on standard cultivation media, such as Mueller Hinton agar or tryptic soy agar, are used. The entire effluent from the test filter, as well as aliquots of dilutions of effluent, are passed through an analysis membrane. Post-challenge recoveries done using analysis membranes allow for assay of the entire effluent so that even a single microorganism in the effluent is detected.

Occasionally, other bioburden organisms may be substituted as the challenge organism. *B. diminuta* is an ideal challenge organism for several reasons. It was a process isolate recovered from effluents (1) following filtration through 0.45 μm-rated filters, which were at one time considered sterilizing-grade filters. *B. diminuta* is an aerobic, asporogenous (does not form spores), gram-negative bacillus that is roughly 0.3×0.8 μm in size, and when grown under limiting growth conditions exists in a single cell form.

Important variables in a microbial retention test are (*1*) culturing conditions of the microorganisms for use as challenge: culturing conditions affect size, shape, and aggregation state of the bacteria. (*2*) the challenge load or bioburden: the specification for challenge level, a minimum of 1×10^7 cfu/cm^2 of filter area, is far in excess of the bioburden routinely encountered in typical pharmaceutical process streams. Challenge concentrations higher than 1×10^8 cfu/cm^2 are not recommended as caking/plugging from excessive bioburden can occur; (*3*) process considerations during testing: passage of microorganisms through partially retentive filters may be enhanced by application of high pressure; temperature may affect fluid viscosity as well as viability and growth rates of microorganisms; and (*4*) solution characteristics of the carrier fluid: the pH, ionic strength, osmolarity, and presence of additives such as surfactants, especially in the case of simulated process streams, may influence microbial retention; it is necessary to ensure that these variables are controlled in the test, to assure reproducibility of test results.

Factors that could potentially affect microbial retention include filter type, eg, structure, base polymer, surface modification chemistry, pore size distribution, and thickness; fluid components, eg, formulation, surfactants, and additives; sterilization conditions, eg, temperature, pressure, and time; fluid properties, eg, pH,

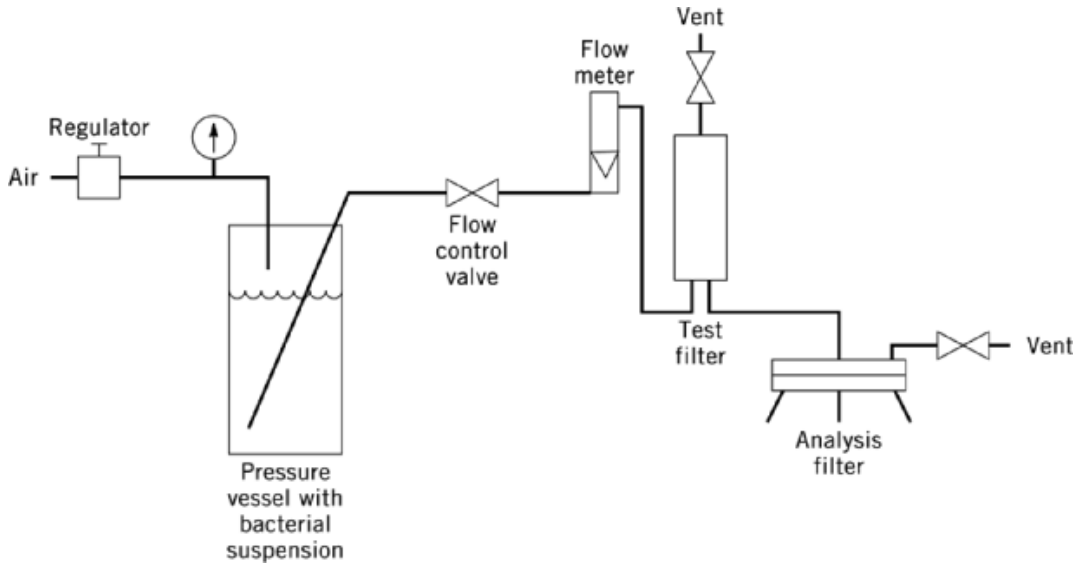

Fig. 1. Schematic of an experimental liquid bacterial setup for evaluation of bacterial retention.

viscosity, osmolarity, and ionic strength; and process conditions, eg, temperature, pressure differential, flow rate, and time.

The efficiency of the filter is evaluated in terms of the titer reduction or log reduction value (LRV). The titer reduction is the ratio of the number of microorganisms in the input suspension to the number of organisms in the effluent. Similarly, the LRV is the $\log_{10}$ of this ratio. The ratio of the difference between the numbers of challenge microorganisms recovered upstream and downstream of the test filter to the average total challenge received by the filter provides an indication of the removal efficiency of the filter, ie,

$$\text{removal efficiency, } \% = \frac{\text{average total challenge} - \text{average total recovery}}{\text{average total challenge}} \times 100$$

When the filtrate is sterile, the number 1 is substituted for the average total recovery and the value is expressed as greater than the calculated value.

Although *Brevundimonas (Pseudomonas) diminuta* (ATCC 19146) is most commonly used for sterilizing-grade filter validation, in certain applications other bacteria are used. For example, when it is necessary to demonstrate removal of mycoplasma in applications involving sera and tissue culture media, membranes having a smaller pore size rating, eg, 0.1 μm, are frequently used. For these membranes, *Acholeplasma laidlawii* may be employed for validation purposes (9).

1.2.1. Integrity Testing

The only test of a filter's ability to remove bacteria is demonstration of its performance characteristics by bacterial-retention testing. However, a bacterial challenge is a destructive test and precludes subsequent use in a filtration operation. Therefore, filter manufacturers provide validation documentation for a filter with correlation of microbial removal to other nondestructive physical integrity tests. Examples of nondestructive tests most commonly used in the industry are bubble-point determinations, pressure hold testing, and forward

(diffusive air) flow measurements. These methods have the advantage of serving as in-process checks on the integrity of the sterilizing membrane while ensuring proper pore size. In the preparation of parenterals current practice is to subject the filter assembly to a nondestructive integrity test both prior to and after completion of the filtration operation.

The industry-accepted integrity tests are performed by applying gas, eg, air or nitrogen, pressure to a wetted filter and monitoring the air flow. These tests, applicable to both hydrophilic and hydrophobic membrane filters, include forward flow, bubble point, and pressure hold. The forward flow test quantitatively measures the diffusive flow as well as flow through any open pores in a wetted membrane filter. The test is performed by wetting a membrane filter and applying a predetermined constant pressure. The test pressure is established for a particular membrane filter by the filter manufacturer. The diffusional gas (air or nitrogen) flow rate as well as the flow through any open pores is measured through the wetted membrane. The gas flow is usually defined in units of mL/min. The filter is integral if the forward flow value is lower than the manufacturer's specified value. The bubble point is qualitative and is dependent on the observation downstream of bubbling through the largest pores of a wetted filter. This test is usually employed for small surface area filters such as membrane disks. To perform the visual bubble point test, the filter membrane is first wet using an appropriate solvent, then air or nitrogen pressure is slowly applied until the wetting fluid is expelled from the largest pores and gas bubbles appear from a submerged tube in a downstream collection vessel. The pressure hold is a modified form of upstream forward flow testing and involves the measurement of decay in pressure over a specified time period for a given filter assembly and wetting fluid. Because the test is performed upstream, the downstream sterile connections are not disturbed. The advantage of this test is that it can be performed after sterilization of the filter assembly, as well as pre- and post-filtration. The filter housing is pressurized to the test pressure specified by the filter manufacturer, then the filter is isolated from the pressure source. The diffusion of gas across the wetted membrane is measured as a decay in pressure over a specified period of time. The pressure hold and forward flow tests are related through the ideal gas law (10). Automated filter integrity test instruments are available in order to provide reproducibility.

1.2.2. Validation Considerations

The need to validate all processes related to the manufacturing of pharmaceutical and biopharmaceuticals has been well established in good manufacturing practices (GMP) regulations and various other guidelines. Filtrative particle removal may be attributable to other mechanisms in addition to direct interception or size exclusion, including, for example, adsorptive particle arrest. It is therefore necessary to validate filter performance as the efficiency of the given filter is dependent on the physical, eg, viscosity and temperature, as well as the chemical, eg, presence of surfactants, composition of the suspending fluid. Microbial retention is required to be demonstrated under simulated pharmaceutical conditions in order to document the performance claims of the filter (3).

Validation of sterile filtration processes is required to be carried out under worst-case conditions. Typically, multiple filter lots (usually three) are challenged with the product under actual or simulated process conditions. A membrane filter having a pore size that allows penetration by the challenge organism is also included as a control. Most commonly, an 0.45 μm-rated filter is included when validation of 0.2 μm-rated sterilizing-grade filters are tested, and should show incomplete retention of *B. diminuta* (1). Essentially, the criteria for the selection of the challenge organism is that it should be small enough to challenge the retentivity of the sterilizing-grade filter and simulate the smallest organism that may occur during production.

If a specific organism has been identified as a routine bioburden, this organism may be substituted for *Brevundimonas (Pseudomonas) diminuta* (ATCC 19146). Ideally, validation experiments are conducted in the product under conditions that closely simulate process conditions. This is, however, not always possible. For example, prior to any microbial retention study it is necessary to determine viability of the test organism in the test fluid. If the product affects the viability of the test organism, as, for example, in the case of cytotoxic drugs,

an appropriate substitution is essential. A placebo, ie, a formulation designed to simulate the actual product having the ionic strength, osmolarity, viscosity, surface tension, and other attributes equivalent to the product, but which does not contain the active drug substance should be used. Alternatively, the native formulation may be modified, ie, the active ingredient is present but the bacteriocidal component removed/neutralized/diluted to ensure no effect on microbial viability. Similarly, if other process conditions affect the viability of the test organism, appropriate modifications are essential. Simulation of other process conditions is also essential. For example, hydraulic process conditions should be simulated during the bacterial challenge to assess any effect on the filter relative to its ability to retain bacteria. Such conditions include maximum differential pressure and pulsing. The pressure differential across the test filter should meet or exceed the maximum pressure differential observed during processing, within the design specifications provided by the filter manufacturer. This serves to validate the filter's ability to retain bacteria in the product and provide a sterile effluent up to or beyond the maximum process pressure differentials.

1.2.3. Sterilization Considerations

In sterile filtration processes the downstream side of the filter must be sterilized and must remain sterile during the entire process. Presterilized (gamma-irradiated) filters may be available or alternatively, filters may be sterilized by the user. The most common method of sterilization is by steam under pressure. The sterilization process must be validated to ensure that sterile conditions are met for a given system. Methods that involve steam are validated through the use of thermocouples and/or biological indicators to ensure sterilization of the system. The filters may be sterilized in an autoclave. Alternatively, the sterilization may be undertaken in-place, called sterilization in-place (SIP) or *in situ* sterilization. Most filter manufacturers provide protocols and recommendations for these procedures. Small-volume systems tend to be autoclaved; larger systems are typically SIP. Minimally, a temperature of 121°C is used for autoclave sterilization; more commonly, a sterilization temperature of 125°C is employed. Temperatures in excess of 125°C may affect the plastics used for filter construction, thereby affecting the physical integrity of the membrane. Standard precautions must be followed during autoclaving: the system must be purged of air to achieve reliable sterilization, and the filter assembly wrapped using a porous barrier to ensure steam penetration. It is critical that excessive differential pressures are not created during the autoclaving which would result in damage to the membrane. These same considerations are all the more relevant during *in situ* sterilization. Additionally, in SIP operations the condensate must be drained throughout the steam cycle to prevent accumulation. This is achieved by keeping the drains and steam traps partially open during the steaming cycle. After the steam valve is closed, a noncondensable gas, such as air or nitrogen, is introduced into the housing to prevent formation of a vacuum on the upstream side of the filter. If it is necessary that the system be completely dry prior to use, the air–nitrogen flow can be continued until the system is dry and cool to operating temperature.

Other methods of sterilization may also be used. Irradiation has the advantage of assuring sterility without any residual gas components. However, several polymers used in filter manufacture may have limited resistance to irradiation sterilization. As with any other process, it has to be validated for sterility. Spores of *Bacillus pumilus* are the indicator organisms for validation of radiation sterilization. Another method is gas sterilization using a gas such as ethylene oxide. For successful sterilization of filters by ethylene oxide, the filters must be dry and wrapped so as to allow penetration of the gas into the matrix or the filter. In addition to the health and safety concerns associated with ethylene oxide gas itself, by-products such as ethylene chlorohydrin and ethylene glycol may be generated which also constitute health hazards. If ethylene oxide is used, appropriate venting is necessary following the sterilization cycle; however, in spite of venting there are concerns that some of the by-products may remain in the filter matrix. The sterilization method must be validated. The indicator organism recommended is *Bacillus subtilis* spores.

1.3. Sterile Filtration of Gases

Primary applications for sterile gas filtration are the sterilization of fermentor inlet air, fermentor vent gas, vents on water for injection tanks, and vacuum break filters during lyophilization. Operational and process considerations apply. Typically, the membrane in gas filtration applications is a hydrophobic membrane, eg, PVDF or PTFE, although there are applications in which the liquid (condensate) in the system is well controlled and hydrophilic membranes may be used. The inherent hydrophobicity of membrane filters used for fermentor air sterilization allows these filters to remove bacteria completely from inlet air, even when exposed to moisture (11). The effluent for gas filtration applications is typically filtered at the 0.2-μm level.

Verification of the microbial retention efficiency of the membrane filters may be undertaken using either liquid or aerosol challenge tests. A liquid challenge test is more stringent. Furthermore, this test can provide retention information for process conditions such as extreme moisture after sterilization or air entrained with water drops. A liquid challenge is performed using a protocol similar to that described for liquid filtration.

Aerosol challenges may be conducted using essentially a test setup composed of three components: the nebulizer, mixing chamber, and a sampling system. An applicable system was first described in 1978 (12). A schematic representation of an aerosol challenge setup is provided in Figure 2. The challenge microorganism is aerosolized using a nebulizer. The aerosol is then mixed with compressed dry air to ensure that the monodispersed microbial challenge to the filter is delivered as a dry aerosol, rather than as microdroplets. Sampling may be done using a vacuum switch device that alternates between the upstream and downstream impingers, and a split-stream liquid impingement method. Any excess air flow not collected by the impingers is vented through an exhaust filter located upstream of the filter. Following the challenge, the buffer from the impingers located upstream and downstream of the test filters are assayed using standard microbiological methods. The dual impingers allow for precise determination of the actual challenge level for each test filter and calculation of the efficiency of titer reduction of the input challenge level.

Filters for use in sterile gas filtration must conform to standards similar to those mandated for sterile liquid filtration. Nondestructive integrity tests may be applied. The tests are performed by wetting the filter with an appropriate solvent, commonly 60/40 isopropyl alcohol/water for hydrophobic membranes, and applying air or nitrogen gas at a preset pressure.

2. Filtration of Virus Removal

2.1. General Principles

Filtration was traditionally used for the removal of bacteria and mycoplasma from biologicals that were heat labile. Advances in filtration technology have resulted in the availability of filtration devices for applications involving removal of viruses. The virological safety of biologicals and biopharmaceuticals is a key consideration in their manufacture. Much of the concern regarding viral contaminants in therapeutic agents centers around blood and blood products as well as biopharmaceuticals which have a blood or tissue component to their production. Human viruses of greatest concern have included human immunodeficiency virus (HIV), hepatitis B virus, hepatitis C virus, cytomegalovirus, and parvovirus. Nonhuman viruses such as bovine viral diarrhea virus are of concern if raw materials derived from these animals are incorporated into the production scheme. Viruses represent a diverse group which include enveloped and nonenveloped viruses, and ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) viruses of various sizes. The focus herein is on removal of viruses from fluids.

Methods used to ensure virological safety are briefly classified as either virus inactivation or virus removal methods. The former includes chemical inactivation, pasteurization, uv inactivation, and solvent–detergent and ion-exchange (qv) chromatography (qv) (13–16). Whereas these methods can be very effective depending on the inactivation process and the nature of the product, there are limitations to the application of these methods.

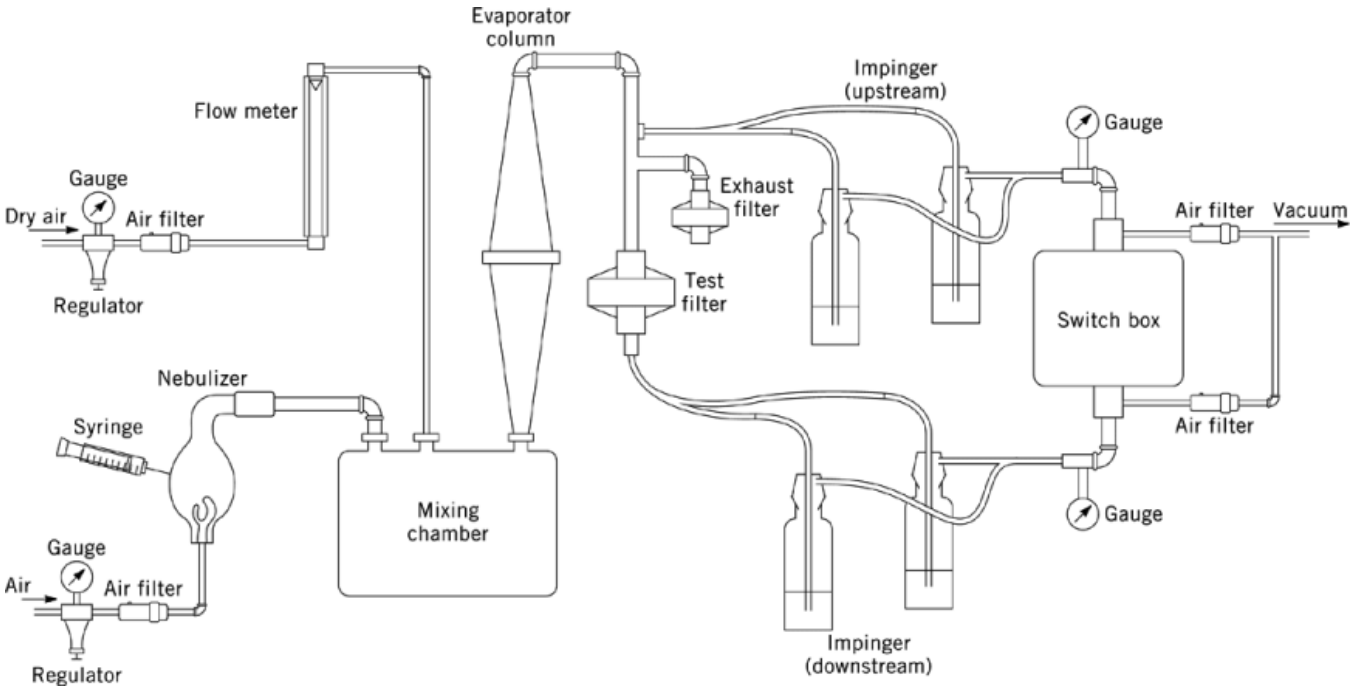

Fig. 2. Schematic of an experimental aerosol bacterial challenge setup for evaluation of bacterial retention.

Stabilizers, sometimes used to ensure that the biological activity is not compromised during the treatment, need to be removed from the final product. Heat treatment can denature certain proteins (17, 18). Processes such as solvent– detergent, chemical treatment, and uv irradiation are often not uniformly effective against all viruses, especially enveloped ones.

The most desirable mechanism for the removal of viral particles using filtration is size exclusion. However, as in the case of bacterial removal by filtration, other mechanisms may also influence virus removal. These factors can include viral adsorption to the filter surface by electrostatic interactions; changes in pore size characteristics during filtration owing to deposits of material on the membrane surface, eg, development of a gel layer; and the filtration conditions, flow rate, pressure, temperature, etc. For example, adsorption can be a complex relationship between fluid pH, membrane chemistry, and the level of organics or protein in the fluid. Thus the removal of viruses by particle size minimizes many of the variables affecting the level of retention and can be a predictable means of sterilization. Removal of viral particles from fluids by size exclusion is preferable. However, concomitant with the requirement for adequate virus removal there is always the inherent necessity for no significant loss in product concentration and/or activity following filtration processes.

2.2. Virological Safety Considerations

Unlike bacterial removal where the specifications for a sterilizing-grade filter are clearly defined and have been implemented and validated over time, the virological safety of biologicals requires consideration of several complex issues. There are several inherent limitations of viral assays. Viral assays may lack the sensitivity to detect low levels of virus, which, although low, may be of medical concern. Demonstration of quantitative removal of bacterial contaminants can be made as, for example, by assay of the entire effluent post-filtration. It is not feasible to assay the entire volume for viral assays; therefore, proving absolute freedom from endogenous

or adventitious viruses is impossible. Additionally, because of the specificity of virus assays, a different assay must be performed for each virus. Direct testing for the absence of viral contamination from a finished product is not considered sensitive enough for establishing freedom from an infectious virus. Historically, there have been several unfortunate iatrogenic accidents involving virus dissemination via administration of vaccines and blood products. Transmission of Hepatitis A (19), Hepatitis B (20), Hepatitis C (21), and HIV (22) via administration of blood-derived products has been reported.

Minimization of risk of inadvertent exposure to real and theoretical viral contaminants is achieved by incorporation of multiple barriers to virus transmission in an integrated manner. This provides overlapping and complementary levels of protection with different modes of action, and separately derived virus reduction potential (23). Approaches that have been recommended are prevention of access of virus by screening of raw materials/precursors used; monitoring production, ie, adventitious virus testing; and a general evaluation of the manufacturing process, ie, process validation of viral clearance. Establishing the absence of infectious virus in the final product often is not derived solely from direct testing for its presence but also from a demonstration that the purification regimen is capable of removal and/or inactivation of viruses (24). Similar recommendations have also been made in Europe by the Committee for Proprietary Medicinal products (CPMP) (25).

Safety is thus the result of multiple barriers operating in concert. Whereas each approach individually may have limitations, use in an integrated manner provides overlapping and complementary levels of protection. These approaches may provide an effective method of overcoming risk and represent the only feasible approach in the face of theoretical risks which cannot be adequately characterized by classical technology.

2.3. Configurations of Virus Filtration Systems

The two principal membrane filtration systems for the removal of viral particles from fluids are single-pass or direct flow filtration and cross- or tangential flow filtration. In the first, the entire volume is filtered through the membrane filter. Typically, the membrane is either a flat sheet cut into disks or a pleated sheet assembled as a cartridge filter. The pore size of these types of membrane filters are generally between 50–100 nm (26). Pleating of the membrane has been used to increase filter surface area and thus increased volume and flow rate of the filtrate. Typically, filters in this format are designed to be used once.

The advantage of single-pass over cross-flow filtration is that it is an easier system to operate and can be cost effective, particularly if the product to be filtered is expensive, because very little of the initial fluid is lost during filtration. However, because the flow pattern of the fluid is directly through the filter, filter life may be too short for the fluid being filtered. The minimum flow rate needed downstream of the filter must also be considered, especially when there are time constraints to the process. In some situations it may be more advantageous to use a cross-flow system where higher flow rates may be easier to obtain.

In the cross-flow mode, fluid is passed across the membrane surface while a portion of the flow is diverted through the filter (permeate). A portion of flow is returned to the central reservoir as retentate. In this process the volume of fluid in the retentate continually decreases as more of the initial volume is collected as permeate. Viral particles are concentrated in the retentate. The advantage of this process is that the cross-flow across the membrane helps extend filter life by reducing gel layer formation. Typically the filtration systems utilizing cross-flow are either in the tangential-type system where fluid passes between two flat sheets of membrane material (Fig. 3) or consist of hollow-fiber filters where the fluid passes through the middle of hollow tubes (Fig. 4) (see Hollow-fiber membranes). For tangential flow, the membrane pore sizes range from 70–180 KD (27–29), as well as 100 and 300 KD polyethersulfone membranes. Hollow-fiber ultrafilters between 100 and 6 KD have been used for virus removal (30–33). These have been constructed from regenerated cellulose fiber, poly acetalnitrile (PAN), and polysulfone (PS). Both types of filters (tangential flow and hollow fiber) are typically designed for reuse after cleaning and sanitization and thus can be cost effective in terms of the filter cost. On the other hand, these systems may have higher holdup volumes and thus greater loss of production than single-pass systems. The cross-flow system may also be more complex and costly to install. Additionally, it is

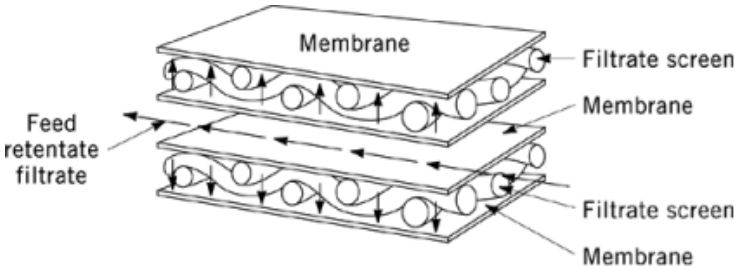

Fig. 3. Diagram of a tangential flow filtration system.

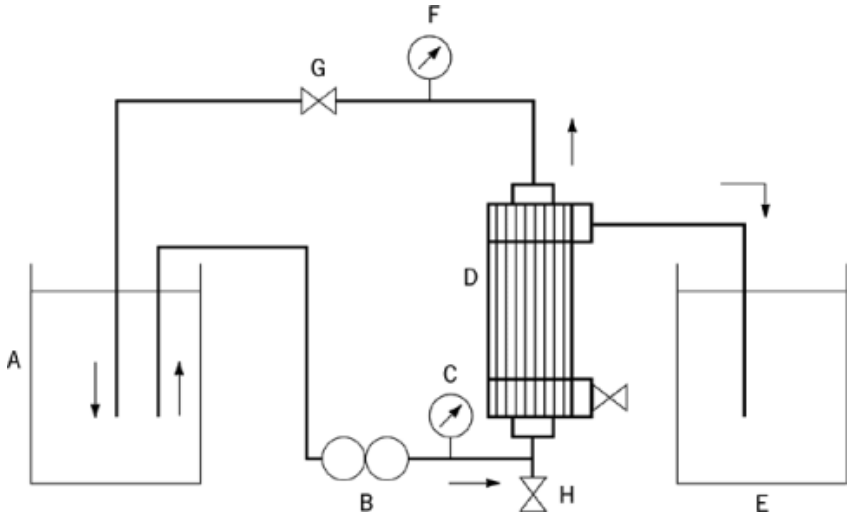

Fig. 4. Diagram of a hollow-fiber ultrafilter filtration system where A corresponds to the retentate reservoir; B, circulation pump; C, pressure gauge at module inlet; D, ultrafilter module; E, permeate reservoir; F, pressure gauge at module outlet; G, value to control module outlet pressure; and H, drain valve.

necessary to validate the filtration process. In the case of reuse of the filter system the cleaning and sanitization procedures also require validation.

2.4. Methods to Detect and Quantitate Viral Agents in Fluids

In order to assess the effectiveness of membrane filtration the ability to quantitate the amount of virus present pre- and post-filtration is critical. There are a number of techniques used. The method of choice for filter challenge studies is the plaque assay which utilizes the formation of plaques, localized areas in the cell monolayer where cell death caused by viral infection in the cell has occurred on the cell monolayer. Each plaque represents the presence of a single infectious virus. Virus quantity in a sample can be determined by serial dilution until the number of plaques can be accurately counted. The effectiveness of viral removal may be determined, as in the case of bacterial removal, by comparing the virus concentration in the input suspension to the concentration of virus in the effluent.

The plaque assay is desirable because it is very sensitive and only detects infectious viral particles. However, there are viral agents which cannot be supported by cell lines. In these cases other methods must be used. The polymerase chain reaction (PCR), which amplifies DNA or RNA from viral agents, can be used to

detect the presence and quantity of viral agents. The amount of RNA or DNA target in the initial sample can be determined by competitive PCR where the quantity of amplified product is compared to a control PCR product where the initial amount of target is known. Quantification is also possible by an end-point dilution method similar to that used to determine a tissue culture infections dose. PCR methods can be very sensitive; however, detection is based on the presence of nucleic acid and consequently the method does not differentiate between an infectious or noninfectious viral particle. In general, the detection of viral agents by either cell culture or PCR assay tends to be virus specific. Therefore multiple assay systems may be needed to detect more than one type of virus.

2.5. Effectiveness of Membrane Filtration

2.5.1. Microfiltration

Various membrane filters have been used to remove viral agents from fluids. In some cases, membranes which have pores larger than the viral particle can be used if the filtration is conducted under conditions which allow for the adsorption of the viral particle to the membrane matrix. These are typically single-pass systems having pore sizes of 0.10–0.22 μm. Under situations which allow optimum adsorption, between 10–10^2 particles of poliovirus (28–30 nm) were removed (34–36). The formation of a cake layer enhanced removal (35). The titer reduction when using 0.10–0.22 μm membrane filters declined under conditions which minimized adsorption. By removal standards, these filters remove viruses at a rate on the low end of the desired titer reduction and the removal efficiency varies with differences in fluid chemistry and surface chemistry of viral agents (26).

Smaller pore size microfilters in single-pass systems which have pore sizes small enough to remove some viruses by size exclusion have been examined (26, 37, 38). Minimum levels of virus removal can be established for these systems if fluid and process conditions are employed which minimize removal of viral particles by mechanisms other than size selection.

Nylon filter membranes have been tested using 47-mm disks of the filter material (26, 37). Influenza A virus (80–120 nm) and HIV (80–100 nm) were removed to below detectable limits in all fluids tested. However, removal of viruses smaller than influenza A virus was not as efficient. Titer reduction results for small (25–50 nm) viruses produced large differences depending on fluid type. The highest titer reductions were observed from high purity water and solutions having low concentrations of protein; smaller titer reductions were observed from solutions containing bovine serum. These results suggest that other factors in addition to size exclusion were enhancing the titer reduction for viruses in the 25–50 nm size range. Diminished adsorptive effects have been observed in the presence of serum or by pretreating a normally adsorbent membrane with serum or gelatin.

A PVDF membrane filter has been shown to remove $>10^6$ particles of virus for viruses >50 nm independent of fluid type (8). Viruses smaller than 50 nm are not removed as efficiently but are removed in a predictable manner which correlates to the virus particle size. The chemistry of the suspending fluid affects titer reduction for viruses <50 nm owing to other removal mechanisms, such as adsorption, coming into play. The effects of these other mechanisms can be minimized by using filtration conditions that minimize adsorption.

2.5.2. Ultrafiltration

Ultrafilters have also been examined for viral removal by size exclusion utilizing tangential flow and hollow-fiber membrane systems (27–32, 35, 36). The titer reduction varies depending on virus size and membrane filter pore size distribution. Removal of poliovirus by a 30,000 molecular weight polysulfone ultrafilter removes $>10^4$ particles of poliovirus in water of various qualities. Tangential flow ultrafiltration has been extensively tested using viruses ranging from 28 nm (Phi X174) to 80–100 nm (Murine leukemia virus) in size using a 70 KD PVDF membrane filter (28) (Table 1). There appeared to be a correlation between virus size and titer reduction. In this study, virus removal appeared to be enhanced in the presence of human serum albumin. Regenerated cellulose fiber (BMM) hollow-fiber ultrafilters from 10–80 nm have been tested using human blood

Table 1. Removal of Viral Particles from Fluids by Ultrafiltration

Filter					
Size reference	Material	Virus (size)	Fluid[a]	Log titer reduction	Reference
500K	PS[b]	MS2 (25 nm)	DI	~1.50	35
300K	ceramic[c]	MS2	DI	4.00	
100K	cellulosic[d]	MS2	DI	>6.00	
			water	>6.00	
30K	PS[e]	polio (28–30 nm)	water	>4.00	
~70 nm	PVDF[f]	Phi X174 (28 nm)	PBS	2.93	(28, 29)
		Phi X174	PBS/HSA	7.42	
		polio (28–30 nm)	PBS	3.1–3.51	
		polio	PBS/HSA	4.2	
		SV-40 (40–45 nm)	PBS	4.89–5.65	
		SV-40	PBS/HSA	>5.7	
		Sindbis (54 nm)	PBS	7.41	
		Reo-3 (78 nm)	PBS	7.18	
		Reo-3	PBS/HSA	>7.6	
		MuLV (85 nm)	PBS/FCS	>6.82	
50 nm	cellulose[g]	HCV (35 nm)	plasma	1.50	32
		HCV	plasma	3.50	
		HCV	plasma	4.00	
		HCV	plasma	>4.00	
105 nm	cellulose[g]	HIV (100 nm)	plasma	>5.77	30
		HIV	plasma	>5.67	
		HIV	plasma	>5.85	
		HIV	plasma	>5.74	
		HIV	plasma	>5.94	
50,000	PAN[h]	polio (28–30 nm)	DMEM-10	4.59	(33, 36)
		phage Tl[i]	DMEM-10	6.27	
13,000	PAN[h]	polio (28–30 nm)	DMEM-10	>6.51	
		phage Tl[i]	DMEM-10	7.55	
6,000	PAN[h]	phage PP7 (25 nm)	DMEM-10	7.62	
		phage Tl[i]	DMEM-10	7.80	
6,000	PS[b]	polio (28–30 nm)	DMEM-10	>6.40	
		phage Tl[i]	DMEM-10	7.99	

[a] DI = deionized water; PBS, phosphate-buffered saline; HSA, human serum albumin; FCS, fetal calf serum; and DMEM, Dulbecco's Eagles minimum essential medium + 10% fetal bovine serum.
[b] Polysulfone hollow fiber.
[c] Tubular.
[d] Hollow fiber.
[e] Polysulfone flat sheet.
[f] Poly(vinylidene fluoride), tangential flow.
[g] Regenerated cellulose hollow fiber.
[h] Polyacrylonitrile hollow fiber.
[i] 50-nm head, 150-nm tail.

borne viruses (30–32) (Table 1). Polysulfone (PS) and polyacrylonitrile (PAN) hollow-fiber ultrafilters have also been tested for their ability to remove viruses between 100–25 nm.

2.6. Integrity Testing

As in the case of bacterial removal, it is necessary to carry out an integrity test on the filter, minimally, following filtration to ensure filter performance. Ideally, the integrity test should be performed both pre- and post-use. This is possible when a nondestructive integrity test method is used. Integrity tests used for virus removal filters include a forward-flow test similar to the test done on bacterial removal filters. This test is nondestructive and amenable to use both pre- and post-filtration. The test is correlated to virus removal by the filter manufacturer. Another nondestructive test is the liquid porosimetric integrity test, also correlated to virus removal. Another integrity test includes a gold particle removability test (GPT). This test is destructive and therefore is applicable only post-use. In general, the integrity test results must correlate with the virus removal claims, as specified by the filter manufacturer.

2.7. Validation Considerations

Mechanisms other then size exclusion may be operative in the removal of viruses from biological fluids. Thus virus removal must be validated within the parameters set forth for the production process and using membrane material representative of the product line of the filter.

The validation study for filtrative virus removal essentially involves challenging (spiking) the product using high titers of infectious virus under conditions that simulate process parameters and quantitating virus in pre- and post-treatment samples. Pre-purification treatments and post-purification modification reactions must also be validated. The choice of virus for validation studies is not as clearly defined as in bacterial filtration where there is an industry-accepted standard. No one viral agent can serve as a generic model virus. Thus, validation studies should be conducted using a panel of viruses that includes known contaminants which may represent identifiable and theoretical risks to product contamination, for example, HIV in the case of blood products; suspected contaminants or model viruses resembling suspected contaminants; or a range of viruses of differing properties which are not themselves considered likely contaminants. In some cases, use of surrogate viruses is necessary as some pathogenic viruses, eg, Hepatitis B and Hepatitis C, are not easily propagated in cell culture. Factors affecting virus clearance results include the choice of model viruses, the appropriateness of the scaled-down version, and the search for process variables which may alter the efficacy of virus inactivation–elimination steps, etc.

The virus reduction factor of an individual purification or removal–inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material divided by the virus load in the post-purification material. A clearance factor for each stage can be calculated and the overall clearance capacity of the production process assessed. Total virus reduction is calculated as the sum of individual log reduction factors. Individual manufacturing steps must possess fundamentally different mechanisms of virus removal or inactivation in order for values to be considered cumulative. Additionally, because viruses vary greatly with regard to inactivation or removal profiles, only data for the same model virus can be cumulative.

A membrane filter which can uniformly remove all viral agents regardless of the size of the viral agent is not available. Part of the difficulty is that the efficient recovery of the biological product diminishes as the size difference between the virus and biological product lessens. Thus a balance needs to be met where virus removal and product recovery are optimized.

Improvements in membrane technology, validation of membrane integrity, and methods to extend filter usage should further improve the performance of membrane filters in removal of viral particles. Methods to improve or extend filter life and increase flow rates by creating more complex flow patterns could possibly be the focus of the next generation of membrane filters designed to remove viral particles.

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