IN-VITRO ACELLULAR METHOD FOR DETERMINING FIBER DURABILITY IN SIMULATED LUNG FLUID

Jon Bauer, Stephanie M. Mattson, and Walter Eastes

- Authors' Addresses
- Objectives
- Scope
- Theory
- Fiber Samples
  - Sample types
  - Preparation of samples
    - Fibers of respirable size (average 1 μm in diameter and 20 μm in length)
    - Discontinuous fiber wool with no binder
    - Discontinuous fiber wool with binder
    - Continuous, uniform diameter fibers
    - Blank sample cassette
  - Measurement of fiber properties
    - Length-weighted diameter distribution
    - Density
    - Chemical composition
- Measurement Apparatus
  - Description
  - Parts list
- Simulated Lung Fluid
  - List of chemicals
  - Preparation procedures
  - Intermediate solution preparation procedures
- Measurement Procedures
  - General
  - Sample loading
  - Measurement by solution analysis
  - Measurement by weight loss
  - Analysis
- Calculation of the Dissolution Rate Constant
  - Calculation of total mass loss
  - Calculation of the dissolution rate constant for uniform diameter fibers
  - Calculation for nearly uniform diameter fibers
  - Calculation for non-uniform diameter fibers when no fibers dissolve completely
  - Calculation for non-uniform diameter fibers when some fibers dissolve
- Future Improvements
  - Fluid composition improvements
  - Improved dissolution kinetics
- References Cited

Authors' Addresses

The printed version of this document is available from the authors:

Dr. Jon Bauer
Johns Manville Corporation
Mountain Technical Center
10100 West Ute Avenue
Littleton, CO 80127
U.S.A.

Dr. Stephanie Mattson
Owens Corning
Science and Technology Center
2790 Columbus Road, Route 16
Granville, OH 43023
U.S.A.

Dr. Walter Eastes
Owens Corning
Science and Technology Center
2790 Columbus Road, Route 16
Granville, OH 43023
U.S.A.

Objectives

Evaluation of fiber toxicity involves a multi-tiered process which begins with short term screening procedures and may proceed ultimately to long term in-vivo chronic assays (McClellan et al., 1992). A critical step in this process is the assessment of the potential durability of a respired fiber in the lung based on the reaction of the fiber with a model lung fluid. These types of studies have been done in many laboratories throughout the world (Bauer et al., 1988; De Meringo et al., 1994; Förster, 1982; Klingholz and Steinkopf, 1982; Leineweber, 1982; Potter and Mattson, 1991; Scholze and Conradt, 1987), but at present there is no standard protocol to assure the accuracy and reproducibility of test results. The intent of this document is to give a working set of procedures that can be readily implemented by any interested laboratory.

This document provides a method to measure the rate of degradation of vitreous fibers that is relevant to their likely persistence in the lung. In doing so, this method scales the degradation process to a single parameter that is a useful approximation to long fiber endurance in the lung. It is proposed that the constant velocity dissolution rate constant k_{dis} for a fiber in simulated extracellular lung fluid be adopted as such a measure.

The intent of this document is to provide procedures which give reliable and reproducible results from laboratory to laboratory. It is also intended that these procedures could be readily implemented in most chemical laboratories without resort to unique or unduly expensive equipment or analytical facilities. The procedure identifies and controls key variables involving sample preparation and characterization, test system design, fluid composition, reaction kinetics, sample analysis, and calculation of rate constants.

Dissolution (mass loss) is a key process that can be related to the persistence of fibers (Eastes and Hadley, 1995) and to lung disease observed in exposed laboratory animals (Eastes and Hadley, 1996). Dissolution is especially important for fibers longer than 15 to 20 μm which reside primarily in the extracellular fluid lining the surface of the alveolar epithelium. This environment is primarily responsible for the degradation of those fibers that are sufficiently long to resist envelopment and translocation by alveolar macrophages. Persistence of these long fibers has been correlated with the onset of respiratory disease, including cancers, in laboratory animals (Davis, 1994).

It is recognized that not all fibers dissolve in the same way. For example, some fibers appear to dissolve nearly congruently, whereas others dissolve incongruently, that is, different components of the fiber are removed at different rates. Furthermore, processes other than mass loss like breakage may occur. However, to a reasonable degree, these various processes may be accounted for by a single dissolution rate constant. It gives a useful and understandable approximation to a complex process.

Another objective of this method is to evaluate dissolution relative to the chemical composition of the glass comprising the fiber and to some extent to any manufacturing process details that might impact the rate at which the glass degrades. It is not specifically intended to evaluate the effect of coatings or other additives to the fiber surface, although such information may be derived from the method if desired.

Scope

This procedure may be used for all commercial and experimental inorganic synthetic vitreous fiber (SVF) materials (TIMA, 1991) composed of individual fibers whose geometry can be adequately described as that of a homogeneous, solid right circular cylinder. It has been tested for borosilicate glass wool fibers over a wide range of composition (Mattson, 1994), for a variety of conventional commercial rock and slag wool fibers, and for refractory ceramic fibers and found to reproduce well the rate at which long fibers dissolve in the rat lung (Eastes et al., 1995), to correlate well with the disappearance of long fibers in the rat lung (Bernstein et al., 1996; Eastes and Hadley, 1995), and lung disease in rats (Eastes and Hadley, 1996). However the procedures given here for obtaining a dissolution rate constant from the measured data do not apply without modification to fibers that are structurally or chemically inhomogeneous. Also, it is known that the dissolution rate measured by these procedures does not adequately reproduce the disappearance of the long fibers of certain new rock wool fibers characterized by 20% or more by weight Al_2O_3 and 42% or less SiO_2. These are active areas of research.

In the strict sense, this method is not applicable to dissolution of asbestos or organic polymer fibers because they may be crystalline rather than vitreous or glassy, because their morphology is different from that of SVF, and because they dissolve so slowly. A modification of this method (Potter and Mattson, 1991) has been used in a comparative sense to approximate biodegradation rates of these fibers relative to SVF. The modification involves a different treatment of surface area changes with time.

Theory

Long fibers residing in the extracellular fluid of the lung are typically widely separated from one another and bathed in a well buffered fluid that is in close contact with the blood stream. Under these conditions, the dissolution products are expected to be rapidly removed from the vicinity of the fibers so that they do not interfere with the subsequent dissolution of the fibers. In the in-vitro measurement specified here, this rapid removal of dissolution products and preservation of the fluid composition and pH is effected by causing the simulated lung fluid to flow through a mat of the fibers at a high but controlled flow rate.

The kinetics of fiber dissolution under these conditions has been described previously. (Leineweber, 1982; Scholze, 1988). At a sufficiently high flow rate, the rate of mass loss of a fiber is proportional to the fiber surface area A,

$$dM/dt = -k_{dis} A, \qquad \textbf{(1)}$$

where k_{dis} is the dissolution rate constant, assumed independent of time, conventionally expressed in $ng/cm^2/hr$. For uniform diameter fibers dissolving congruently from an initial mass M_o and initial diameter D_o, a solution to Eq. (1) is

(2)

$$1-(M/M_o)^{1/2}=\frac{2k_{dis}t}{D_o\rho},$$

where ρ is the initial fiber density. A consequence of Eqs (1) and (2) is that the diameter D of a fiber decreases at a constant rate according to

$$D=D_o-\frac{2k_{dis}t}{\rho}. \quad \textbf{(3)}$$

The fibers in most samples to be evaluated, as in actual fiber aerosols, do not all have the same diameter, as assumed in Eqs. (1-3). One remedy to this situation is to calculate the mass loss at each experimental time for each fiber diameter measured in a sample of the initial fibers, assuming some value for the dissolution constant. The assumed dissolution constant is then varied until adequate agreement with the experimental value is obtained.

The method just outlined can be done analytically in a simple form if it can be assumed that only an insignificant number of fibers have completely dissolved at the times measured. In this case, Eq. (2) may be solved for the mass M of each fiber with initial diameter D_o. The total mass $\sum M$ of all of the fibers is found to obey the equation

$$\frac{\sum M}{\sum M_o}=1-\frac{4vt<D>}{<D^2>}+\frac{4v^2t^2}{<D^2>} \quad \textbf{(4)}$$

where $\sum M_o$ is the total initial mass, v is the dissolution velocity,

$$v=\frac{k_{dis}}{\rho}, \quad \textbf{(5)}$$

v = {k sub dis} over rho , $<D>$ is the length weighted, arithmetic average initial fiber diameter, and $$<D^2>$ is the average value of the square of the initial diameters. This latter quantity is given in terms of the length weighted, arithmetic standard deviation of the initial fiber diameter σ by

$$<D^2>=<D>^2+\sigma^2. \quad \textbf{(6)}$$

Equation (4) is a quadratic equation for v for a measured initial fiber diameter distribution and total fiber mass at each time. It may be solved for v at each time and k_{dis} obtained by Eq (5).

No assumptions are made in this method about nature of the glass structure; No distinctions between network and network modifying components of the glass are made. The measure of dissolution is the total mass loss from the fiber sample over time. Unlike for crystalline materials, it is difficult at best to determine reliably where each of the many glass components resides in the structure and therefore whether they are network-formers or not. The character of the dissolved species is also important and is not affected by the glass structure. However, such a distinction may not be necessary as the total mass loss has proven adequate to explain major differences in persistence and biological activity of a wide variety of SVF compositions in laboratory animals (Eastes and Hadley, 1996).

Fiber Samples

Sample types

In principle, this method applies to SVF in any form including both continuous filament and discontinuous (wool) forms. Since manufacturing processes may have some effect on the dissolution rate, samples for

measurement should be produced by the same or similar process as that creating fibers comprising the actual exposure in question.

Preparation of samples

Fiber samples are prepared for dissolution rate measurement by shortening them to an average length of 100 to 500 μm to insure uniform packing in the test cassettes, removing any non-fibrous particulate material or shot, suspending the resulting fibers in distilled water, and vacuum filtering them into a mat on a filter in a cassette. If any oils or organic contaminants are suspected, the fiber samples should be washed thoroughly with a non-polar solvent like cyclohexane.

The sample cassette containing the filter is described further on. It is first dried for 2 d under vacuum at 50°C followed by 2 d in a vacuum desiccator at room temperature, and then dried to constant weight in a glove box purged with dry N_2 and containing $CaSO_4$ desiccant at room temperature. The sample cassettes are weighed on an analytical balance to an accuracy of 0.1 mg. The weighed cassettes are kept in sealed plastic bags or in a desiccator until they are needed.

The steps needed to prepare a suspension of shortened fibers from various forms of the original fiber material are given below. This suspension is then dispersed in an ultrasonic agitator if needed to disperse them and vacuum filtered to form the mat. The cassette containing the filtered fibers is dried for 2 d under vacuum at 50°C and then again to constant weight in a glove box or desiccator with $CaSO_4$ desiccant at room temperature. The weighed cassette containing the fibers is kept in a sealed plastic bag until it is inserted into the dissolution measurement apparatus.

Fibers of respirable size (average 1 μm in diameter and 20 μm in length)

These fibers require no further shortening for in-vitro dissolution measurement. At least 500 mg of sample should be made available. They should be dispersed in distilled water in a blender for 15 s or with an ultrasonic agitator so that they will be evenly deposited on the filter. Such fibers are not the best samples for dissolution rate measurement, since the fibers are so short and the resulting mats very dense. When the fibers are too close to each other, they interfere with the dissolution of the neighboring fibers in a way that would not be expected to happen to inhaled fibers in the lung.

Fluid percolation through these mats may also be non-uniform resulting in preferred channeling through certain areas. While these effects may not be able to be completely eliminated, they may at least be mitigated by making the fiber dispersion on the filter as uniform as possible and by using an initial mass as small as possible to keep the mat thin.

Discontinuous fiber wool with no binder

If not already small enough, the wool is cut into 1 cm pieces with scissors or a scalpel. Shot or non-fibrous particulate, if present, is separated by chopping about 3 g of the pieces with distilled water in a blender and separating the material that suspends from that which does not suspend with agitation. The shot is heavy and falls away as the fibers are chopped into short lengths in the blender. Shot is undesirable as it provides mass which may be unaccounted for in the rate calculations. The suspension of fibers from which the shot has been removed, or that originally contained no shot, is dispersed for 15 s in a blender.

At this point, the sample should be examined using an optical or scanning electron microscope to assure that it is free of non-fibrous particulate. A problem, however, exists if the shot is particularly fine. This situation may necessitate longer settling times in water under quiescent conditions, followed by decantation of the suspended fraction. This procedure may need to be repeated several times. It is recommended that the sample or a representative aliquant be reexamined after each step to determine the presence of shot. No more than three attempts at refinement should be made. Caution should be exercised to avoid prolonged exposure to water, particularly if the fiber sample is suspected to be non-durable. The practitioner will need to judge when the benefits derived from a more shot-free sample are compromised by potential loss of mass and increased fiber surface area.

Discontinuous fiber wool with binder

It has been shown that many types of organic binders have little effect on the overall dissolution process over a major portion of the fiber's lifetime. The binder is therefore removed by low temperature ashing. Removal by high temperature ignition may change the dissolution rate of the fiber by annealing the structure. In any case, ashing does not remove the inorganic components of the binder. It is preferable to obtain samples without binder. After the binder is removed, the wool is then chopped in the blender as just described.

Continuous, uniform diameter fibers

Continuous filament of 2 to 10 μm diameter fibers are first cut into 1 cm sections and then chopped in a blender for 15 s with distilled water. This procedure will make fibers with lengths in the range of 100 to 1000 μm, which are easily dispersed on the supporting filter and will not create interferences or flow problems in the test cells.

Blank sample cassette

During every series of fiber dissolution rate measurements, a blank cassette should be operated in the same apparatus. This cassette has the same construction, filter, and support pads, and is connected to the same tubing with the same solution flow rate as the fiber filled cassettes, but it contains no fibers. The blank cassette is used to provide a pH measurement when no fibers are dissolving and to provide effluent solutions to be analyzed for the background concentrations of each element. These background concentrations are subtracted from the concentrations measured for dissolving fibers in the calculation of total fiber mass remaining.

Measurement of fiber properties

The calculation of the dissolution rate constant from the measured amounts of fiber components dissolved requires knowledge of a number of physical and chemical properties of the initial fibers. These properties and methods to determine them are described here.

Length-weighted diameter distribution

An accurate measurement of the length-weighted fiber diameter distribution is required to evaluate the dissolution results. For continuous, uniform diameter fibers, it is sufficient to measure 40 to 60 diameters and to

assume a Gaussian or normal distribution described by the average and standard deviation of the diameters measured. For non-uniform diameter fibers, the length weighted diameter distribution should be measured (Koenig et al., 1993) using at least 400 fibers. If a bivariate length and diameter measurement is available for the tested fibers, it may be used to construct the length weighted quantities (Koenig et al., 1993).

It is advisable to maintain a separate count for non-fibrous particulate (NFP). As noted in the previous section, this mass is normally accounted for in rate constant calculations so it is important to determine the particle fraction of NFP and at least an estimate of its mass contribution before the test is begun. The procedures described earlier in this document should eliminate most of the larger shot particles. However, depending upon their shape, some finer particles may escape and be carried over with the fiber fraction. If the dimension of this particulate is on the order of the diameter of the fiber or larger, then only a small amount can be tolerated. If the fraction is greater than 1% on a particle count basis, then the sample should be rejected and the elutriation procedures described earlier initiated or repeated until the above specification is met. If the particulate is much finer than the fibers, it is likely to have a much higher specific surface area and may make a significant contribution to the mass loss early on in the test. No specific rejection criteria can be given as the influence of the NFP will depend on a number of factors including composition, shape, and internal porosity. Caution should be exercised when using samples containing fine NFP.

Density

An estimate of the fiber density to three significant digits is needed. A number of pycnometric or density fluid column methods are available to measure it to the required accuracy. Some difficulties may occur when attempting to measure densities of very thin or short fibers, due to the impact of residual air or even Brownian movement. In such cases, good judgement should be exercised in terms of the minimum fiber dimension that provides a reliable result.

Chemical composition

The composition of the fibers, expressed as weight percent oxide, is needed for the major elements in order to estimate the dissolution of components not measured in the solution. The major elements are ordinarily Si, Al, Ca, Mg, Na, K, B, and Fe, and any others that account for at least 98% of the fiber. Many standard methods for fiber chemical analysis are suitable for this determination.

Measurement Apparatus

In-vitro measurement of fiber dissolution rate in an acellular medium is accomplished by exposing the fiber sample to a simulated extracellular fluid under controlled conditions and monitoring the mass lost via analysis of the collected leachate. The system presented here is designed to provide uniform flow of fluid through the sample, minimal contamination of the fluid by the containment devices, stability to temperature and pH, and efficient collection of fluids for subsequent analysis. While the description is fairly detailed in regard to parts and connections, allowances can be made for design variations and other materials as long as they result in no compromise to the stated tolerances and specifications.

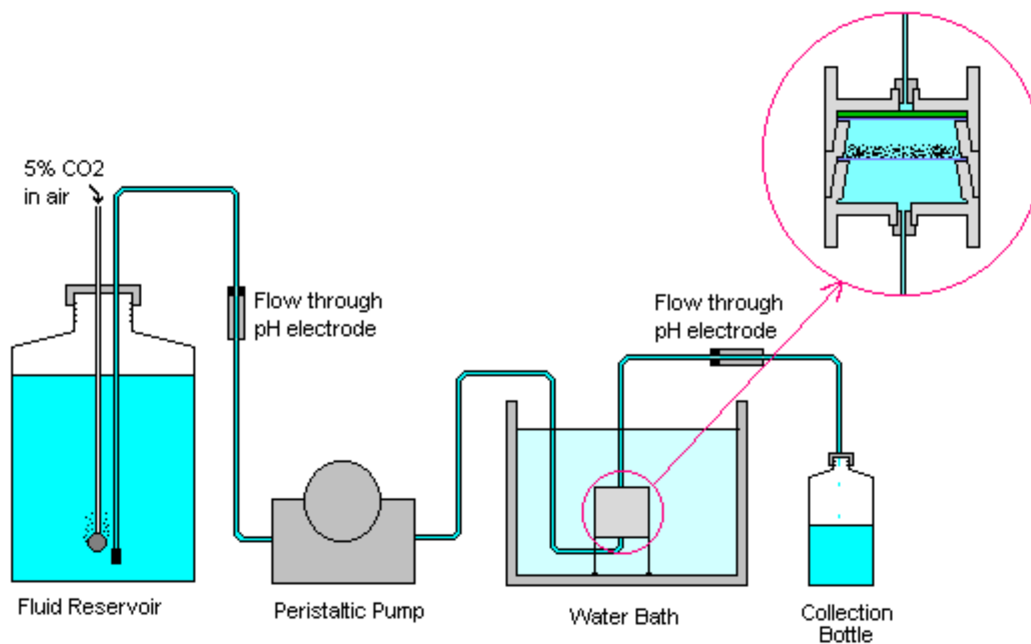

FIGURE 1. Schematic diagram of the dissolution rate measurement apparatus.

Description

A diagram of the in-vitro dissolution rate measurement system is shown in Figure 1. It begins with a 20 liter polycarbonate carboy containing the simulated lung fluid (left side) connected to a pH electrode then to a multiple line peristaltic pump, then to the sample cassette, which contains a mat of fibers on a filter, then to another pH electrode and finally to a collection bottle. The carboy is connected to a cylinder containing 5% CO_2 in air or in N_2. The first pH electrode is a check of the system, and the second is used to insure that the flow rate is high enough to maintain the pH. A standard pH electrode is fitted into a nylon Cajon T with adapters. These adapters may be either Cajon or homemade with PTFE tubing to adapt to the small tubing. In-line pH electrodes are also available with a small internal volume. The pH must be measured in a closed system since CO_2 is evolved into the atmosphere with an open system and the pH rises quickly. A water bath is used to maintain the sample cassettes at 37.0±0.5^{o}C.

The testing is done using a mat of fibers contained in a slightly modified 37 mm air monitoring cassette from Millipore with polycarbonate filters with pores of 0.1 or 0.4 μm and a plastic support pad. The 0.1 μm size should be used only when the loss of thin fibers is a concern. The filters and pads may be obtained from Nuclepore. The three major pieces of the cassettes are held together by friction. They are further clamped together by an assembly of plastic plates and steel bolts, washers, spacers, and nuts during the test. This assembly helps keep the cassettes upright and keeps the tubing below it from kinking. It also allows the cassettes to be removed without affecting the other cassettes that are under test. The cassettes are connected to a T that links them to the simulated lung fluid or to distilled water.

The collection bottles at the end are used to monitor the flow rate. The tubing from the carboy to the collection bottles must be very impermeable to CO_2 and it should have a small internal volume. Tygon tubing was found to yield an unstable pH. The following two different types have been found to work: PTFE tubing (0.064 inches OD, 0.032 inches ID) and PharMed tubing (3/16 inch OD, 1/16 inch ID or 1/4 inch OD, 1/8 inch ID for fast

flow). The PTFE microtubing is fairly flexible but needs a more flexible sleeve to link to connectors, such as 3/16 inch OD, 1/16 inch ID Tygon tubing. Quick-connect Luer miniature plastic fittings from Cole Parmer are useful. In addition to simple fittings, which are attached to the tubing, a Luer type manifold supplies up to ten lines and three-way stopcocks are used to connect to the pH electrodes. The peristaltic pump should have multiple lines and variable speed and be capable of low rpm. The MASTERFLEX L/S system with ten lines and the MASTERFLEX L/S motor with an ISMATEC attachment are suitable. Both systems can have mixed flow rates but the ten line MASTERFLEX unit changes flow rate from the lowest to the next step by a factor of six by changing holders and tubing size. The ISMATEC systems hold eight pieces of tubing, which change the flow rate by a factor of three just by changing the tubing. The motors on the MASTERFLEX pumps run from low rpm (1 to 10 rpm) up to 100 rpm. Other motors have maximum speeds of 600 rpm.

Parts list

The system was designed to have only plastic parts (with a few exceptions) in contact with the simulated lung fluid.
Source carboy, 20 liter polycarbonate
Mixing carboy, 10 or 20 liter polycarbonate with 10 liters marked on the side

Aspirator stone
5% CO_2 in air or in N_2 cylinder
Silicone tubing inside carboy weighted with stainless steel nuts
pH electrodes and meter
Tubing, PTFE and Tygon or PharMed
Connectors, male, female, lock ring, 2-way and 3-way connectors, plugs, bulkhead mounts
Manifolds and 3-way stopcocks
Peristaltic pump
Collection bottles, 1 liter
Effluent sample bottles, 50 ml polyethylene
Solution collection bottles, 1 or 2 liter polycarbonate or polyethylene
Blender with small cups
Glove box and antistatic device
Analytical balance
Dry nitrogen cylinders to flush glove box
Desiccant, calcium sulfate
Desiccator for sample storage
Water bath with heater and circulator, plastic balls to cover the surface, and algicide
Sample holders, aerosol cassettes, polycarbonate filters, plastic support pads, clamping devices
Vacuum oven
Vacuum desiccator
Vacuum pump
Chemicals, see Simulated Lung Fluid below, some kept as solids, some as solutions (some of these need dark containers)
Top loading balance for measuring solids
Graduated cylinders for measuring solutions
Magnetic stirrer and stirrer bars, glass coated preferred

Simulated Lung Fluid

The solution described here was selected because it is flexible and easy to use while still providing an environment that is relevant to the extracellular fluid in the lung (Eastes et al., 1995).

The composition of the fluid is given in Table 1. A list of chemicals needed and instructions for preparing this solution are given in the following paragraphs.

List of chemicals

Sodium chloride
Ammonium chloride
Calcium chloride dihydrate
Sodium dihydrogen phosphate monohydrate
Concentrated sulfuric acid, 98%
Glycine
Sodium citrate dihydrate
Solution of 37% formaldehyde and 15% methanol in water
Sodium carbonate
Cylinder of 5% carbon dioxide in air or in dry nitrogen.

TABLE 1. Solution Composition in mM.

Na^+	150.7
Ca^{2+}	0.197
NH_4^+	10
$H_2CNH_2CO_2H$ (glycine)	5.99
H_2CO (formaldehyde)	67
CH_3OH (methanol)	20
Cl^-	126.4
SO_4^{2-}	0.5
HCO_3^-	27
HPO_4^{2-}, $H_2PO_4^-$	1.2
$[HOC(CH_2CO_2)_2CO_2]^{3-}$ (citrate)	0.2
bubbled with 5 % CO_2 in air or in N_2	

Preparation procedures

The following procedure is used to prepare the simulated lung fluid. It makes use of some intermediate solutions, the directions for preparing which are given further on.

Add a teflon coated stirrer bar to about 9 l of distilled water in the mixing carboy at room temperature in a laboratory fume hood. Start stirring and add the following reagents in order. Weigh solids on a top loading balance to an accuracy of 0.01 g and measure solutions using 25 and 50 ml graduated cylinders. Wait for solids to dissolve before adding the next ingredient.

50 ml ammonium chloride solution
67.80 g sodium chloride
17.70 g of sodium bicarbonate
6.29 g sodium carbonate
25 ml sodium dihydrogen phospate solution
25 ml sodium citrate solution
4.50 g of glycine
25 ml sulfuric acid solution. **CAUTION: SULFURIC ACID IS CORROSIVE TO BODY TISSUES. BE AWARE OF THE SHOWER AND EYEWASH LOCATIONS. WASH ANY AREA OF DIRECT CONTACT WITH COPIOUS AMOUNTS OF WATER.** Measure 25 ml of calcium chloride but do not add it to the solution yet.

Turn on the fan in the hood and put on neoprene apron, goggles, and thick vinyl, butyl nitrile, or neoprene gloves. Measure out 25 ml of the formaldehyde and methanol solution. **CAUTION: FORMALDEHYDE IS AN IRRITANT, A SENSITIZER, AND TOXIC. METHANOL IS AN IRRITANT, A NARCOTIC AND A NEUROTOXIN. THIS SOLUTION IRRITATES THE BREATHING PASSAGES AT HIGH CONCENTRATIONS OF THE VAPOR.** Transfer the solution from the 1 l supply bottles using a medicine dropper to control and direct the flow. Add the solution to the carboy and rinse the graduated cylinder three times with distilled water into the carboy. Add the calcium chloride solution and rinse the cylinder once into the carboy.

Rinse the neck of the carboy and shoulder area above the liquid surface to remove any solids or other reagents. Turn off the stirrer and center the carboy on the stirrer plate. Add distilled water to bring the top of the meniscus to the 10 liter mark on the carboy. Return the carboy to the mixing position and stir for 30 minutes. The solution may then be added to the supply carboys.

Intermediate solution preparation procedures

For each of these solutions, 1 to 2 l is prepared in a volumetric flask and they are stored in 1 to 2 l plastic bottles. Some of these solutions contain nutrients but no biocide and must be examined routinely to be sure there is no biological contamination. Newly prepared solutions must not be added to old solutions, but the bottles emptied, rinsed with bleach and distilled water, and dried before being filled with new solution.

Ammonium Chloride. 214.02 g should be placed in a 2 l volumetric flask. Distilled water is added to fill most but not all of the volume. **CAUTION: THE DISSOLUTION OF AMMONIUM CHLORIDE IS STRONGLY ENDOTHERMIC.** The solution should be gently agitated to dissolve the solid. Then the solution is left to return to room temperature. The remaining water is added and the flask agitated to insure a homogeneous solution. The starting material is somewhat dirty and must be filtered. Use qualitative filter paper with medium porosity to filter the solution into a dark bottle.

Sodium Dihydrogen Phosphate Monohydrate. 66.40 g is placed in a 1 l volumetric flask and distilled water added. This solution is kept in a dark bottle.

Sodium Citrate Dihydrate. 23.60 g is placed in a 1 l volumetric flask and distilled water is added. This solution is also kept in a dark bottle.

Sulfuric Acid. 20.31 g of concentrated sulfuric acid should be added drop by drop to a 1 l flask half filled with distilled water. **CAUTION: CONCENTRATED SULFURIC ACID IS VERY CORROSIVE TO ALL**

BODY TISSUES. IT ALSO HAS AN EXTREMELY LARGE HEAT OF SOLUTION WITH WATER. WATER CAN REACH THE BOILING POINT AND SULFURIC ACID CAN BE SPLATTERED IN THE AREA IF A SMALL AMOUNT OF WATER IS ADDED TO SULFURIC ACID OR A LARGE AMOUNT OF ACID IS ADDED TO WATER. Approximately 13 ml should be poured into a small beaker in the hood. The residual acid should be added to the waste container for simulated lung fluid together with one rinsing. The solution should be allowed to return to room temperature before the volume is finally adjusted to the mark.

Calcium Chloride Dihydrate. 11.60 g should be placed in a 1 l volumetric flask and water added. If insoluble particles are noted in the solution, it should be filtered.

Measurement Procedures

General

The method involves the flow of simulated lung fluid at 37°C at a controlled rate through a mat of the fibers. The effluent is then analyzed for several of the fiber dissolution products. The total mass of fibers dissolved is determined from the concentration of each of these dissolution products at each time the effluent was sampled. Finally, the dissolution rate constant is obtained from the total remaining mass of the fibers at each time.

Determination of the fiber dissolution rate constant by measuring the diameter change of fibers in simulated lung fluid has been reported (Potter and Mattson, 1991) but is not recommended here. This method is generally less sensitive than what is presented here and is much more time consuming due to the need to mount and to measure many fibers individually.

An important experimental parameter is the flow rate of the simulated lung fluid over the fiber mat. This flow rate must be high enough for the amount of fiber dissolving that the pH does not rise appreciably and the concentration of dissolution products does not rise high enough to affect the dissolution rate or process (Mattson, 1994). On the other hand, the flow rate must not be so high that the dissolution products are so diluted in the effluent that they cannot be analyzed accurately. The concentrations of each dissolution product is affected by the dissolution rate constant, by the mass of fibers filtered into a mat in the sample cassette, by the diameter distribution of the fibers, and by the flow rate. Since glasses with different compositions may have widely different dissolution rates, some preliminary experiments must be done for new compositions. For compositions similar to ones that have already been measured, adjustment of the flow rate and the fiber weight to account for the surface area differences may be all that is required.

For a given fiber mass, the initial fiber surface area may be calculated from the length weighted diameter distribution. Then, for a given dissolution rate constant, the rate at which mass dissolves in solution is given by Eq. (1). From that quantity and the given flow rate, the total concentration of all fiber components initially in the effluent may be estimated. The concentration of the individual elements in the solution may then be estimated from the oxide composition assuming that they dissolve congruently, that is, in proportion to their mass in the original fibers. The calculations just outlined allow the various experimental constraints to be balanced against one another to arrive at a combination of sample weight and solution flow rate to get started.

A convenient way to ensure that the flow rate is high enough so that the dissolution products do not affect the dissolution rate is to monitor the pH at the electrode following the sample cassette. The pH at this location should not be more than 0.2 units higher than that in a blank cell during the first 24 h and thereafter not more than 0.1 unit higher (Mattson, 1994). For glasses containing alkali and alkaline earth oxides that affect the pH when they dissolve, the constancy of pH is evidence that the flow rate is high enough. On the other hand,

refractory ceramic fibers consisting essentially of SiO_2 and Al_2O_3 dissolve with no effect on pH, yet their dissolution is sensitive to flow rate. In such cases, tests of solution concentrations may be needed.

The determination of an appropriate flow rate is fundamentally done by trial and error, however. Unless the dissolution rate is well known and it is desired just to verify it, two or three separate measurements of the dissolution rate constant at widely different flow rates are needed to establish the correct conditions. These conditions are established by plotting the dissolution rate and its standard deviation against the flow rate at which it was measured with both axes on a logarithmic scale. The flow rate is large enough if the dissolution rate constant does not change within the standard deviation as the flow rate increases.

TABLE 2. Estimated solution flow rate to fiber surface area required to achieve dissolution conditions relevant to the lung.

Dissolution Rate	Flow Rate to Surface
Constant [ng/cm^2/hr]	Area Ratio [μm/s]
< 100	0.01
100 - 1000	0.05
1000 - 10 000	0.5

An estimate of the minimum flow rate to fiber surface area ratio needed to obtain the proper conditions is shown in Table 2. It is based on an extensive series of measurements (Mattson, 1994) and may be used as a guide to get started. The flow rate (volume per time) to fiber surface area has the dimensions of length per time and is given in μm/s here, but cm/hr is also common.
This procedure does not require the use of replicate samples, but some replicate measurements are recommended to evaluate the system. If replicates are done, then the average (arithmetic mean) of the dissolution rate constants from each replicate should be reported along with the standard deviation and the number of replicates.

Sample loading

The simulated lung fluid is pumped into the cassette at room temperature at which the reaction rate is small. When the cassette is full it is placed in the bath and this defines the start time.

Filling the cassette is a touchy process. It is difficult to use a syringe without breaking the filters. Therefore the line from the solution carboy is used to fill and rise the cassettes. A fair amount of bubbles is produced as the simulated lung fluid exsolves dissolved gasses. The solubility of gasses is inversely proportional to temperature and directly proportional to the pressure. Since there is a pressure drop after the peristaltic pumps, these bubbles can be trapped behind the polycarbonate filters where they can cause a vapor lock or trap enough vapor to dry out the fiber mat. Bubbles may be reduced by tapping the cassette and by allowing space above the fiber mat for bubbles to accumulate so that they are not forced into the fiber mat.

The aerosol cassettes are convenient in that they are easily handled (without the plastic and steel assembly) and allow the sample to be kept reasonably secure after filling and during the test. They are also transparent and can show any problems with bubbles or with precipitates or contamination of the simulated lung fluid by various organisms. These latter problems are infrequent. The buildup of bubbles is more common and can be seen under the fiber mats to allow steps to be taken to free them.

Measurement by solution analysis

After the sample cassettes containing the weighed fibers are loaded into the apparatus, the measurement consists of monitoring the pH of the effluent to insure its constancy as previously described, measuring the volume of solution that has passed through the cassettes, and collecting samples of the effluent solution at intervals for analysis. Effluent samples are collected directly from the outflow tubing with measurement of the volume of solution collected. At the same time, the total volume of solution passed since the last sampling (including the volume of the effluent sample) is noted. The samples can be frozen immediately and stored in a freezer until they are analyzed, or they can be stored in darkness and analyzed within about one month.

A minimum of five to eight effluent samples should be taken and analyzed at times well spaced over more than half of the fiber lifetime, that is, the time it would take a fiber of the average diameter in the sample to dissolve. If the fibers dissolve slowly, that is, have dissolution rate constants less than 100 ng/cm^2/hr, or are thick, larger than 5 μm in diameter, it is usually sufficient to take five samples over a much smaller fraction of the fiber lifetime, as little as 2 or 3%. In this case it should be verified that the dissolution rate constant computed for the latter samplings varies randomly, and is not increasing or decreasing significantly with time. If the latter situation occurs, then more samples must be taken to adequately follow the dissolution. If the dissolution rate varies only slightly with time, then it can be described adequately as an average dissolution rate constant. If the dissolution varies greatly with time, then the fiber dissolution cannot be described with a dissolution rate constant.

Measurement by weight loss

Measurement of dissolution by directly measuring the loss in weight may be done as a check on the solution analysis results or, in rare cases, when it is not possible to analyze for the glass components in solution. Sufficient fiber mass must be used to enable an accurate weight difference measurement.

At the end of the desired dissolution time, the sample cell is switched to distilled water to rinse the salty simulated lung fluid away for several hours. These steps are easily accomplished without affecting the other samples being tested. The dissolution rate in distilled water is approximately 1/10 that in simulated lung fluid for most fibers, and the lower temperature also decreases the reaction with water. Thus the mass lost during rinsing is considered negligible.

The cell is removed from its bracket, the exterior is cleansed with distilled water, and the interior water is removed by suction filtration. The sample is then dried and weighed in the same way as it was when it was prepared, as described in previously.

Analysis

Chemical analysis of the effluent samples may be done by inductively coupled plasma arc or atomic absorption spectroscopy, for which standard methods are available. Normally Si, Ca, Mg, K, Al, and B are determined, depending on which of these elements are present in significant amounts in the fibers. Some elements, such as Na, are not useful to analyze because they have a high concentration in the simulated lung fluid. Silicon analysis alone is not recommended as a sufficient measure of glass dissolution. It is important that the frozen samples be allowed to defrost and equilibrate to room temperature for 24 h or more to obtain accurate analyses of some elements, especially Si and B (Mattson, 1994).

Calculation of the Dissolution Rate Constant

The first step in calculating the dissolution rate constant from the measured data is to compute the total mass loss from the dissolving fibers from the concentrations of each cation analyzed in the effluent solution. Then there are several methods available to compute the dissolution rate constant from the mass loss as a function of time. Each of these steps is described separately. These methods generally produce a set of estimates of the dissolution rate constant, one at each time the effluent was sampled and analyzed. The average and standard deviation of this set of estimates is reported along with an identification of the calculation method used to obtain them.

Calculation of total mass loss

All of the methods for calculating the dissolution rate constant require the total mass of fibers remaining at each time sampled, by computing the mass loss from the concentrations of cations in the sampled effluent. The first step in this process is to convert all elemental concentrations to elemental oxides using standard gravimetric conversions. The oxides are those which are conventionally used to describe the initial chemical composition of the fiber. If C_{ik} is the concentration of component i, expressed as the mass of the oxide per volume of solution at time t_k (where the initial time is t_0), then the total mass of oxide i remaining at time t_n is

$$M_i(t_n) = W_i M_o - \sum_{k=1}^{n} (C_{ik} - B_{ik}) V(t_k), \quad \textbf{(7)}$$

where M_o is the initial mass of fibers in the cassette, W_i is the weight fraction of oxide i in the fibers from chemical analysis, and $V(t_k)$ is the solution volume that passed between time t_{k-1} and t_k. The B_{ik} are the concentrations of oxide i in the blank cassette effluent at approximately the time t_k. The total mass remaining at time t_n is the sum of the $M_i(t_n)$ over all oxides

$$M(t_n) = \sum_i M_i(t_n). \quad \textbf{(8)}$$

However, since not all components dissolving from the fibers are normally analyzed, the missing ones must be estimated from those measured. To make this estimate, it is usually assumed that Al_2O_3, if not analyzed, dissolves at the same rate as SiO_2 and that Na_2O and others not analyzed dissolve at the same rate as all measured oxides except SiO_2 and Al_2O_3. (The proportionality of Al_2O_3 to SiO_2 is often an inaccurate assumption, and it is recommended that it be analyzed directly.) For example, if $M_x(t_n)$ is the mass remaining of the sum of all analyzed oxides except those of Si and Al, then the estimated mass of Na_2O remaining is

$$M_{\mathrm{Na}}(t_n) = (W_{\mathrm{Na}}/W_x) M_x(t_n), \quad \textbf{(9)}$$

where W_{Na} is the weight fraction of Na_2O in the original fiber and W_x is the weight fraction of the sum of all oxides except those of Si and Al.

Calculation of the dissolution rate constant for uniform diameter fibers

If the fibers initially have the same diameter $D sub o$, then Eq. (2) may be solved for k_{dis} at each time sampled. The average and standard deviation of these estimates of k_{dis} are reported.

Calculation for nearly uniform diameter fibers

Fiber samples made from continuous filament may not be as uniform in diameter as required for the calculation just described. In this case, the calculation may be done assuming that the diameters are normally distributed. Detailed equations for this calculation have been given (Potter and Mattson, 1991).

Calculation for non-uniform diameter fibers when no fibers dissolve completely

If the fiber diameters are so widely distributed that neither the uniform nor the normal approximations are valid, as is usually the case for fibers prepared from production wool, then it may still be possible to compute the dissolution rate constant from the weight loss data without resorting to an iterative procedure. If most of the fibers are so thick that only an insignificant number of them have dissolved completely at the times sampled, then Eq. (4) may be used for each time.

Calculation for non-uniform diameter fibers when some fibers dissolve

If none of the previously described situations apply, then an iterative method may be used, as outlined in an earlier section. A value for the dissolution rate constant is assumed, and the total mass fraction remaining is computed for each fiber diameter measured in the initial distribution by Eq. (2). The sum of the mass fractions remaining for each diameter fiber is the calculated total mass fraction remaining for the assumed dissolution rate constant. The calculated mass fraction remaining is then compared with that observed at each sampled time. The value of the dissolution rate constant that minimizes the sum of the squares of the differences between the calculated and observed mass fractions remaining is reported.

Future Improvements

There are a number of ways that this protocol might be improved in the future so as to better achieve the objective of accurately measuring fiber dissolution as it would happen to long fibers inhaled into the deep lung. The present protocol has been shown to approximately reproduce both the mechanism and the dissolution rate of a series of fibers in vivo (Eastes et al., 1995) as well as to represent the overall lifetime enough to explain the presence or absence of disease in chronic animal studies (Eastes and Hadley, 1996). Thus any improvements to the protocol must produce the same results as the present protocol for those fiber compositions against which it has been validated in vivo. An improved protocol might, however, improve the agreement between in-vitro in-vivo measurements for new fiber types or better take into account more details of the dissolution process in vivo.

One type of fiber for which the present method is known not to adequately reproduce the behavior of long fibers in the lung is the so-called "high alumina rock wool" fibers, which consist of 20% by weight or more of Al_2O_3 and 42% or less SiO_2. It is thought that Al and perhaps Si dissolving from these fibers impedes their dissolution in vitro in a way that does not happen in vivo. This problem is an active area of research.

Two areas in which improvements can be foreseen for this method are the simulated lung fluid composition and the kinetics of the dissolution reaction.

Fluid composition improvements

The composition of the simulated lung fluid in this protocol was chosen to be the same as the composition of the actual lung fluid in all components that are expected to influence the mechanism or the rate of fiber dissolution. Therefore the pH was adjusted to the value of 7.4 (Ganong, 1973), and the ionic strength in vitro was chosen to approximate that measured in vivo. On the other hand, the concentration of Ca^{2+} in the solution is somewhat less than in the lung to enable determination of small amounts of Ca dissolved from fibers, because it is expected that this difference would not influence dissolution. Furthermore, the in-vitro solution lacks proteins, enzymes, and surfactants found in the lung. In the future it may be found that the absence of certain components may play a

role in the dissolution of new fibers not previously tested. Or it may be found that the agreement with in-vivo could be improved with a closer simulation of lung fluid in vivo. Whenever these changes in the fluid are validated in vivo, they will be incorporated into this protocol.

Improved dissolution kinetics

The zero order rate law of Eq. (1) that is used to interpret all of the measured fiber mass loss is merely an approximation of a more complex process. As more information becomes available about the details of the in-vivo dissolution process, it may be appropriate to incorporate it into the interpretation of the experimental results. Of course, the desired parameter is a single number that can be related to the time a fiber is effectively present in the lung. Various details about the actual dissolution mechanism need to be included in the evaluation of the in-vitro dissolution results only to the extent that they more accurately describe this effective fiber lifetime. Inhomogeneous fibers that are composed of two or more parts with different compositions or fibers that, by virtue of their production process, contain regions with different structure are examples of fibers for which the zero order rate law is not a good approximation.

References Cited

Bauer, J. F., Law, B. D., and Roberts, K. A. 1988. Solubility and durability of man made mineral fibers in physiological fluids. Presented at TAPPI Non-Wovens Conference, Nashville, OH. Available from the author.

Bernstein, D. M., Morscheidt, C., Grimm, H.-G., Thévenaz, P., and Teichert, U. 1996. Evaluation of soluble fibers using the inhalation biopersistence model, a nine-fiber comparison. *Inhal. Toxicol.* 8:345-385.

Boffetta, P. 1994. Biopersistence of respirable synthetic fibers and minerals: The point of view of the epidemiologist. *Environ. Health Perspect.* 102(Suppl. 5):11-13..

Davis, J. M. G. 1994. The role of clearance and dissolution in determining the durability or biopersistence of mineral fibers. *Environ. Health Perspect.* 102(Suppl. 5):113-117.

De Meringo, A., Morscheidt, C., Thélohan, S., and Tiesler, H. 1994. In vitro assessment of biodurability: Acellular systems. *Environ. Health Perspect.* 102(Suppl. 5):47-53.

Eastes, W., and Hadley, J. G. 1995. Dissolution of fibers inhaled by rats. *Inhal. Toxicol* 7:179-196.

Eastes, W., and Hadley, J. G. 1996. A mathematical model of fiber carcinogenicity and fibrosis in inhalation and intraperitoneal experiments in rats. *Inhal. Toxicol.* 8:323-343.
Read this paper or Download it (57 kB).

Eastes, W., Morris, K. J., Morgan, A., Launder, K. A., Collier, C. G., Davis, J. A., Mattson, S. M., and Hadley, J. G. 1995. Dissolution of glass fibers in the rat lung following intratracheal instillation. *Inhal. Toxicol.* 7:197-213.
Read this paper or Download it (57 kB).

Förster, H. 1982. The behaviour of mineral fibres in physiological solutions. In *Biological Effects of Man-Made Mineral Fibres, Proceedings of a WHO/IARC Conference*, Vol. 2, pp. 27-59. Copenhagen: World Health Organization.

Ganong, W. F. 1973. *Review of Medical Physiology*, 6'th ed., pp. 16, 537. Los Altos, California: Lang.

Klingholz, R., and Steinkopf, B. 1982. The reactions of MMMF in a physiological model fluid and in water. In *Biological Effects of Man-Made Mineral Fibres, Proceedings of a WHO/IARC Conference*, Vol. 2, pp. 60-86.

Copenhagen: World Health Organization.

Koenig, A. R., Hamilton, R. D., Laskowski, T. E., Olson, J. R., Gordon, J. F., Christensen, V. R., and Byers, C. D. 1993. Fiber diameter measurement of bulk man-made vitreous fiber. *Analytica Chimica Acta* 280:289-298.

Leineweber, J. P. 1982. Solubility of fibres in vitro and in vivo. In *Biological Effects of Man-Made Mineral Fibres, Proceedings of a WHO/IARC Conference*, Vol. 2, pp. 87-101. Copenhagen: World Health Organization.

Mattson, S. 1994. Glass fibers in simulated lung fluid: Dissolution behavior and analytical requirements. *Ann. occup. Hyg.* 38:857-877.

McClellan, R. O., Miller, F. J., Hesterberg, T. W., Warheit, D. B., Bunn, W. B., Kane, A. B., Lippmann, M., Mast, R. W., McConnell, E. E., and Reinhardt, C. F. 1992. Approaches to evaluating the toxicity and carcinogenicity of man-made fibers: Summary of a workshop held November 11-13, 1991, Durham, North Carolina. *Reg. Toxicol. and Pharmacol.* 16:321-364.

Potter, R. M., and Mattson, S. M. 1991. Glass fiber dissolution in a physiological saline solution. *Glasstech. Ber.* 64:16-28.

Scholze, H., and Conradt, R. 1987. An in vitro study of the chemical durability of siliceous fibres. *Ann. Occup. Hyg.* 31:683-692.

Scholze, H. 1988. Durability investigations on siliceous man-made mineral fibers, a critical review. *Glastech. Ber.* 61:161-171.

TIMA, 1991. *Man-Made Vitreous Fibers: Nomenclature, Chemistry and Physical Properties*. Alexandria, VA: North American Insulation Manufacturers' Association.

First Page || Next Page