A Novel Method for Determination of Sterility of Microcapsules and Measurement of Viability of Encapsulated Organisms

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A method of determining the viability of microencapsulated microorganisms (Bacillus Calmette Guerin) is reported. This method was also used to measure the effectiveness of aseptic production of microcapsules in maintaining the interior of the microcapsules free from contamination by microorganisms. This method is advantageous over conventional plating methodology, as plating can only determine external contamination of microcapsules and similar devices. It involves the detection of $^{14}CO_2$, which is generated by the metabolism of ^{14}C-labeled fatty acid in the growth medium by encapsulated microorganisms. The method depends on the semipermeable nature of the microcapsule walls, which allows passage of ^{14}C-palmitic acid and $^{14}CO_2$. BCG organisms encapsulated within an alginate–polylysine–alginate microcapsule (5–15 μm) (1) were shown to be viable, and no contaminating organism(s) was present. Methods suitable for the aseptic production and freeze drying of alginate–polylysine–alginate BCG microcapsules, which retain the viability of the organisms, are reported.

KEY WORDS: microencapsulation; determination of sterility of the interior of microcapsules; Bacillus Calmette Guerin (BCG); viability of encapsulated microorganisms; freeze drying; microcapsules; aseptic processing of microcapsules; alginate/polylysine microcapsules.

INTRODUCTION

An air-atomization method used to produce small-sized microcapsules (5–15 μm) containing encapsulated Bacillus Calmette Guerin (BCG) organisms has been reported (1). This method, which is an adaptation of the method of Lim and Sun (2), involves spraying a suspension of BCG organisms in a sodium alginate solution into a calcium chloride solution to form temporary calcium alginate microgel droplets. A Turbotak is used in conjunction with an infusion pump and a compressed air source to atomize the sodium alginate–BCG suspension. The temporary microgel capsules are subsequently cross-linked with polylysine to form permanent membranes. The microcapsule size is dependent on the conditions of operation of the Turbotak and the concentration of the sodium alginate solution (1).

The overall goal of this research is to produce targetable BCG microcapsules for the treatment of lung cancer and tumor metastasis in the lung. BCG, an attenuated strain of *Mycobacterium bovis*, is known to be a potent immune adjuvant (3). Passive targeting to the lung can be achieved by intravenous injection of particles in the size range 5–15 μm which become entrapped in the fine capillary beds of the lung. The microcapsules (1) were designed to target the BCG organisms, which themselves have the dimensions 2.36 × 0.47 μm, to the lung.

The BCG microcapsules must be axenic, that is, the microcapsules must contain only live BCG organisms and no other contaminant organisms. Moreover, the BCG organisms must remain viable after the encapsulation process, and the microcapsule membranes must be semipermeable to allow nutrients to diffuse in and excretion products to diffuse out. There is a direct relationship between BCG vaccine viability and its therapeutic efficacy in treating superficial bladder cancer (4). Another study, using a murine bladder tumor model, showed that the viability of BCG organisms is important in determining the efficacy of intravesical BCG therapy (5). Mackaness *et al.* noted that the immunogenicity of a BCG preparation was related directly to the viability of the vaccine (6). Most methods of microencapsulation involve harsh conditions which might prove harmful to microorganisms, such as the use of organic solvents and heating during the processing. The calcium alginate gel method of Lim and Sun (2) has been successfully used to encapsulate live islets of Langerhans. This method is not harmful to the live cells, as they are protected within the gel formed in the initial stage. However, the shearing forces applied during the atomization process employed to produce small microcapsules could potentially be harmful to the BCG organisms.

Axenic production of microcapsules containing live organisms is problematic, as end-point sterilization is potentially destructive to BCG, and aseptic processing must therefore be performed. The sterility of the outside of the microcapsules can be tested by conventional plating methodology; however, it is difficult to assess both the viability of the encapsulated organisms and the lack of contamination within the microcapsules. Disruption of the microcapsules to test viability and sterility could be destructive to the cells and adds another step where contamination may be introduced. Production of $^{14}CO_2$ from ^{14}C-labeled fatty acid is used as a measure of BCG viability and axenic production of microcapsules without disruption of the microcapsules is reported here. The microcapsules are inoculated into sealed vials containing growth medium. Living mycobacteria utilize ^{14}C-labeled fatty acid which is present in the medium and produce $^{14}CO_2$. $^{14}CO_2$ is detected by a Bactec 460 instrument and is reported as a growth index (7–9). The microcapsule membrane must be semipermeable, as ^{14}C-labeled fatty acid and $^{14}CO_2$ must pass across the membrane.

This method of determining the presence of viable microorganisms within microcapsules has wide pharmaceutical implications in the aseptic production of controlled and targeted drug delivery systems such as microcapsules, liposomes, nanoparticles, emulsions, erythrocyte ghosts, and implantable devices that cannot undergo end point sterilization. The assurance that the inside of these delivery systems is free from contamination by microorganisms has been problematic to date.

The BCG microcapsules must be suitable for storage prior to use. Freeze-drying, as a method of choice to prepare

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Table I. Growth Index of Encapsulated BCG With and Without Using Aseptic Technique

	Growth index (GI)				
Aseptic technique	1 day	2 days	5 days	7 days	9 days
Yes	—	47	350	817	999
No	999				

Table III. Growth Index of BCG-Encapsulated Microcapsules With and Without the Addition of Panta Plus

	Growth index (GI)				
Panta plus	1 day	3 days	6 days	8 days	10 days
No	11	50	395	864	999
Yes	11	67	586	999	

a stable product, was also investigated. The encapsulated BCG organisms must survive this processing.

MATERIALS AND METHODS

Materials

Sodium alginate (medium viscosity), poly-L-lysine (MW 38,500), sodium citrate, calcium chloride, sodium chloride, bile salts, and citric acid were obtained from Sigma Chemical Company. Magnesium sulfate, potassium phosphate monobasic, ammonia water, and glycerin were obtained from Fisher Scientific. Lactose, iron ammonium citrate, and asparagine were obtained from J. T. Baker Chemical Co. BCG (Tice strain) was obtained from the Institute for Tuberculosis Research, University of Illinois at Chicago. Millex-GS 0.22-μm filters were obtained from Millipore. Bactect 12B medium and Panta Plus were obtained from Becton Dickinson.

Production of Axenic Microcapsules

All solutions to be used, calcium chloride, sodium chloride, sodium citrate, and bile salts, are autoclaved using a Barnstead steam sterilizer (Model C57835) at 121°C for 20 min. The solutions of sodium alginate and polylysine are sterilized by manually passing through a 0.22-μm Millex-GS filter. All the equipment used, including all the glassware and the Turbotak, are sterilized by autoclaving at 135°C for 5 min. The air used to atomize the sodium alginate–BCG suspension is filtered as it passes through the tube which connects it to the Turbotak. A piece of cotton is placed inside the tubing, which is then autoclaved at 135°C for 5 min. The microcapsule production process is performed in a class II laminar flow hood (Nuaire, Model NU-425-300).

Method of Encapsulation

One ampoule of BCG is reconstituted in 0.5 ml of a 0.1% (w/v) aqueous bile salt solution and dispersed in 15 ml of a 1.2% (w/v) aqueous sodium alginate solution. This suspension is infused from a syringe pump (Sage Instrument, Model 355) at 2.2 ml/min into an air atomizing device (Turbotak, Turobotak Inc., Waterloo, Ontario) and sprayed into a pan containing 500 ml of 1.5% (w/v) calcium chloride solution. The air pressure is set at 40 psi. The distance between the orifice of the Turobotak and the pan is fixed at 18 in. The orifice is 1 mm in diameter. The divalent calcium ions cross-link the droplets of sodium alginate on contact to form temporary microgel droplets. The temporary capsules are subsequently cross-linked by mixing with a poly-L-lysine (0.035%, w/v) solution for 6 min to form a permanent membrane. The resultant permanent capsules are collected by centrifugation at 3000 rpm, washed in 30 ml of a 0.9% (w/v) sodium chloride solution, and then suspended for 4 min in 0.03% (w/v) sodium alginate to form a final cross-linked alginate outer layer. The alginate–poly-L-lysine–alginate microcapsules are washed twice with 0.9% sodium chloride. The final permanent microcapsules are then added to 30 ml of 55 m*M* sodium citrate, pH 7.4, for 6 min to liquify the calcium alginate gel in the interior of the microcapsules. The resulting capsules are washed twice in 0.9% (w/v) sodium chloride solution. Microcapsules processed with reagents mentioned above or after washing were collected by centrifugation at 3000 rpm for 3 min.

Test for Viability of the Encapsulated BCG and Aseptic Production of the Microcapsules

Two-tenths milliliter of microcapsules is inoculated into Bactec 12B vials and incubated at 37°C before testing. One-tenth milliliter of antibiotic cocktail (Panta Plus) is added to each vial to inhibit the growth of any contamination bacteria. Other batches without the addition of Panta Plus are tested to determine if the microcapsules are axenic, that is, they contain only BCG and no other contaminating microorganisms. The Bactec 12B medium is an enriched Middlebrook 7H9 broth base. Mycobacterium utilize a ^{14}C-labeled substrate (palmitic acid) which is present in the medium and release $^{14}CO_2$ into the atmosphere above the medium. When the vials containing the 12B growth medium are tested on the

Table II. Growth Index of Different Concentrations of Plain BCG

	Growth index (GI)							
Colony-forming units (CFU)	1 day	3 days	6 days	8 days	10 days	13 days	15 days	17 days
500,000	7	15	433	999				
5,000	4	2	3	19	151	999		
500	5	3	1	2	11	189	789	999

Table IV. Growth Index of Control Groups

Control group	Growth index (GI)
Heat-killed BCG	<10
Plain microcapsules	<10

Bactec 460 instrument, the $^{14}CO_2$ is aspirated from the vials and the radioactivity is determined quantitatively in terms of numbers on a scale from 0 to 999; 999 is the maximum value which the instrument measures. These numbers are designated the growth index (GI). Any GI reading of 10 or more is considered a positive result, that is, viable organisms are present. Control groups which consist of heat-killed BCG microorganisms, empty microcapsules, and BCG alone are tested. The GI of samples prepared with and without using the aseptic technique described above is also tested. At least three batches were tested for each group. The microcapsules are plated on tryptic soy agar medium (soybean–casein digest agar medium) and incubated at 37°C overnight to test for outside contamination of the microcapsules. No contamination was found.

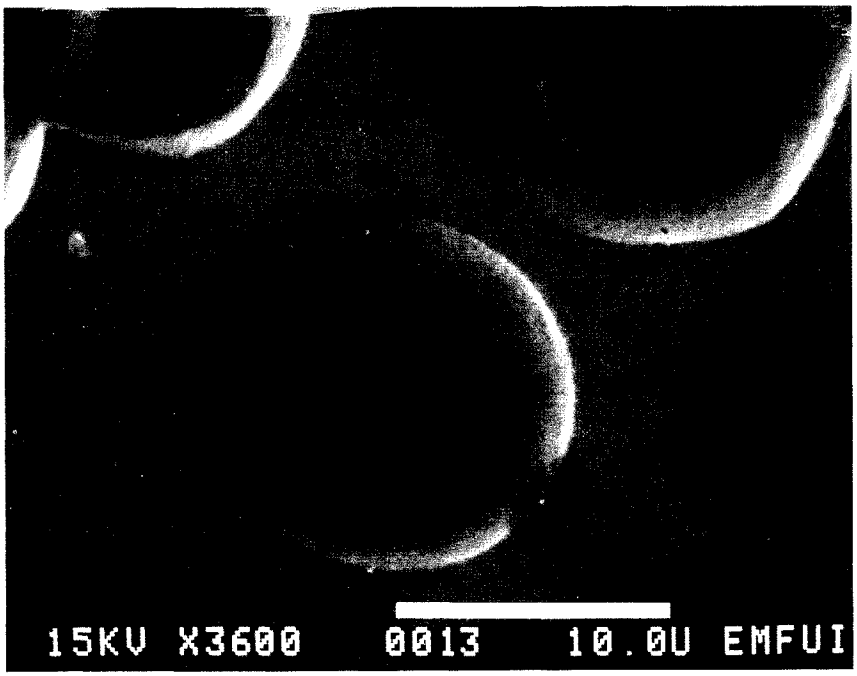
Freeze-Drying of the Microcapsules

BCG-encapsulated microcapsules are washed twice with distilled water and freeze-dried using a Labconco freeze-dryer. The microcapsules are frozen at −70°C overnight and then freeze-dried (−60°C, 10 μm Hg) for 72 hr. Another batch of BCG-encapsulated microcapsules is mixed with the formula reported by Rosenthal before freeze-drying (10). This formula consists of two solutions: solution I, which contains an 18% (w/v) lactose solution; and solution II, which contains asparagine, citric acid, potassium phosphate monobasic, magnesium sulfate, glycerin, iron ammonium citrate, and ammonia water. The viability of the BCG organisms in different batches of freeze-dried microcapsules is tested.

RESULTS AND DISCUSSION

Table I shows the GI of the BCG-encapsulated microcapsules with or without aseptic technique as determined by the Bactec 460 instrument. BCG is a slow-growing organism compared to other bacteria. For example, BCG organisms require at least 3 to 4 weeks to form colonies on agar plates (11). A dramatic increase in GI within the first day would indicate that the sample is contaminated by other bacteria. The batch produced without using aseptic technique generates a GI of 999 when incubated overnight. The contamination in this batch was confirmed by gram staining and by subculturing on a blood agar plate. The contaminating organisms were found to be gram negative and there was growth on the blood agar plate after incubating overnight. In contrast, the GI of the batch produced using aseptic technique rises slowly and reaches a maximum reading of 999 in 9 days. At least three batches were tested for each group and the data were consistent. The results are shown for only one batch. The Bactec methodology described is an extremely fast and efficient method to determine the viability of encapsulated organisms and the sterility of microcapsules. BCG organisms require at least 3 to 4 weeks to form colonies on agar plates, whereas with the Bactec method metabolism is detected within 2 days. The instrument is automated and therefore data collection is extremely efficient compared to conventional plating technology.

Utilization of the fatty acid by the BCG organisms and release of CO_2 into the atmosphere above the medium are determined by the metabolism rate of the organisms and are



not directly related to the number of organisms present. Table II shows results obtained for the GI of BCG organisms alone. The increase in GI is not linearly related to the number of organisms inoculated for the samples tests: 500, 5000, and 500,000 organisms. The rate of metabolism of organisms is not directly proportional to the number of organisms in any biological system. The BCG organisms alone give GI data which increase with time, following the same trend as the axenic BCG microcapsules.

The purpose of the addition of Panta Plus to the growth medium is to reduce the possibility of contamination of the inoculated sample. However, the addition of Panta Plus also reduces the chance of detecting any contamination which may have occurred during the processing of the microcapsules. Table III shows that there is no difference in GI for batches tested with and without the addition of Panta Plus. These results indicate that the microcapsules were produced axenically.

Table IV lists the GI data for the various control groups: heat-killed BCG organisms, empty microcapsules, and BCG alone. The heat-killed BCG and empty microcapsules show no growth regardless of the incubation time up to 21 days when the study was terminated. It can be concluded that the rise in the growth index for the BCG microcapsules is caused by the living BCG organisms. As a result of the extensive processing during microencapsulation, it is unlikely that any BCG organisms are attached to the outside of the microcapsules. To exclude this possibility, batches of aseptically produced BCG microcapsules were plated on a modified semi-solid medium reported by Rosenthal *et al.* (12). Absence of colony growth over a period of 42 days indicated that no viable BCG organisms were attached to the outside of the microcapsules and that the encapsulated BCG organisms cannot grow through the microcapsule matrix. No BCG organisms were found on the outside of the microcapsules when investigated by SEM. Figure 1 is a SEM showing the surfaces of BCG microcapsules. During the encapsulation process, BCG are entrapped inside the matrix of the microcapsules.

Utilization of ^{14}C-palmitic acid (MW 256.42) by the BCG organisms to produce $^{14}CO_2$ demonstrates that the microcapsule membranes are semipermeable, which is critical to the viability of the encapsulated organisms.

Freeze-drying as a method to preserve the BCG microcapsules may affect BCG viability. Table V shows the results of viability testing of batches of microcapsules with and without using the freeze drying formula for BCG reported by Rosenthal *et al.* (12). The encapsulated BCG organisms require the particular adjuvants contained in the formula for protection during the freeze-drying process. Without the addition of these adjuvants the BCG organisms were apparently killed during freeze-drying, as no GI was detected up to 21 days when the viability study was terminated. The addition of the adjuvants is designed to prevent the formation of ice crystals inside the BCG organisms and preserves viability during the freeze-drying process.

Table V. The Effect of Freeze-Drying Adjuvants on the GI of BCG-Encapsulated Microcapsules

	Growth index (GI)				
Adjuvants	1 day	3 days	6 days	8 days	10 days
Yes	3	3	49	290	999
No	4	2	2	2	3

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