UNIVERSITY OF ILLINOIS

This is to certify that the thesis prepared under my supervision by

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entitled

The Technique of Making Alginate Beads for Microencapsulation of Cells

is approved by me as fulfilling this part of the requirements for the degree of Bachelor of Science in Chemical Engineering.

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The Technique of Making Alginate Beads for Microencapsulation of Cells

By

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Thesis

for the
Degree of Bachelor of Science
in
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## FIGURES

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Microencapsulation involves enclosing cells in semi-permeable membranes. Encapsulation of cells is accomplished by first immobilizing cells in polymer gel beads, then placing membranes around the beads, and then dissolving the polymeric beads. By choosing membranes with selective permeabilities one can effectively control the substances diffusing through the membrane and ensure desired cell environment interaction. The membrane also offers protection to shear-sensitive cells and provides a surface to which anchorage-dependent cells can adhere. Because cells remain encapsulated, they can be easily recovered for analysis.

There are many applications to this technology. One such application, which will be the future study in continuation of this project, is to separate mutant yeast cells that have higher glucoamylase secretion rates than others. Yeast cells are encapsulated individually in microcapsules containing starch solution. Polysaccharide such as starch is too large for yeast cells to digest as nutrient, therefore cells secrete glucoamylase which breaks down starch to glucose, a monosaccharide, for cells to digest and grow. Capsules containing mutant yeast cells with high glucoamylase secretion rates will soon be over-populated with cells because high concentration of glucose induces rapid cell growth. After a time period, capsules are recovered and mutant cells are separated based on the buoyancy density of the capsules.

Four criteria regarding microencapsulation are important and should be addressed prior to the entrapment of cells, especially for the separation of mutant yeast cells described above. It is essential to produce uniform size and reproducible microcapsules since the
The objective of this work is to design an apparatus and select appropriate operating conditions to produce small (100-300 μm), uniform spherical calcium alginate beads (without cell entrapment) at the highest alginate solution flow rate possible. The bead generating apparatus has been successfully built and factors influencing the behavior of the beads were investigated. Bead size as a function of air flow rate and extrusion rate was studied during this project. Factors affecting the formation of the beads such as calcium chloride concentration, sodium alginate concentration, distance between needle and calcium chloride solution, and stirring speed, were also studied.

SURVEY OF LITERATURE

Alginate Gel Entrapment

In recent years, the use of calcium alginate gel matrix for immobilization of cells stands out as the most promising and versatile method. This cell immobilization process can be performed in a single step under very mild conditions and is therefore compatible with most living cells. Calcium alginate beads are formed by dripping droplets of sodium alginate solution and cell suspension mixture into a curing bath containing calcium cations. The droplets form gel spheres instantaneously, entrapping cells in a three dimensional
lattice of ionically cross-linked alginate. There are some characteristics of alginate that should be noticed. Some alginites contain small amounts of polyphenols which might harm sensitive cells. Depending on the application, precautions must be taken in selecting a suitable alginate. Alginate lattice has high porosity which leads to leakage of large molecules like proteins. However, for the purpose of microencapsulation, high porosity of alginate gel is not important since the alginate lattice will be dissolved eventually. The beads can be dissolved easily by immersing the beads in a solution containing phosphate or citrate. Substances such as phosphate or citrate have a high affinity for calcium ions, and will sequester the cross-linking calcium ions, consequently destabilizing the gel. However, by adding polycations such as chitosan or polypeptides, or synthetic polymers such as polyethyleneimine, these alginate complexes will not dissolve in the presence of calcium chelators or anti-gelling cations, and can both stabilize and reduce the porosity of the gel.

Microencapsulation Techniques

Many microencapsulation techniques were studied and compared in preparation for this work. Below is the summary of the work done by different research groups. Most research groups utilized cell immobilization apparatus similar to the one employed in this project; however, the smallest bead size achieved by any groups was around 400 μm [9], [2]. In this work, by using a smaller diameter needle and higher air flow rates, we were able to produce uniform and spherical alginate beads with diameter around 200 μm.

Encapsule

This conventional microencapsulation technology (trade name ENCAPSEL) which involves complexing the polymer, poly-1-lysine (PLL), with sodium alginate was initially developed by Lim and Sun [3]. The modified technique developed by Goosen [4] is
briefly described here. A cell pellet is prepared by centrifuging a suspended cell culture and
decanting the medium. The cell pellet remaining at the bottom of the centrifuge tube is then
resuspended in sodium alginate solution. The alginate-cell suspension is extruded into a
calcium chloride solution. Spherical droplets of this suspension are formed by an air-jet-
syringe pump droplet generator. After the droplets are gelled, they are reacted with an
aqueous PEL solution to form semi-permeable capsule membranes on the surfaces. After
the addition of PEL, the tube is capped and shook to keep the capsules from sticking
together. Contact with sodium alginate solution forms an outer layer on the capsules. The
microcapsules are then washed with CHLS, calcium chloride and isotonic saline. The
interior of the capsules is liquefied with sodium citrate. The microcapsules are washed
several times in saline, then incubated in DMEM medium in 37°C incubator. The method
of making multiple membrane capsules is also described in this work [4].

The molecular weight cut-off point of the capsule membrane can be controlled by:
1. varying the alginate-PEL reaction time and the temperature, 2. varying the Mw of the
PEL, 3. varying the concentration of PEL, and 4. by mixing PEL of different Mw's [5].
The Mw cut-off point of the membrane can be decreased by either increasing the alginate-
PEL reaction time, by decreasing the Mw of PEL (less steric hindrance), or by increasing
the PEL concentration. The study shows an increase in the number of alginate-PEL ionic
interactions causes a decrease in the Mw cut-off point of the membrane [4, p.244]. By
mixing two PEL's of different Mws, membranes with characteristics intermediate to those of
one PEL Mw can be achieved. The physical strength is also dependent on the Mw of PEL and
the alginate-PEL reaction time. A longer reaction time and lower Mw of the PEL will yield
a stronger membrane [4, p.248].
Chitosan-Alginate Complex

In this microencapsulation technique employed by Daly and Knorr [6], the chitosan-alginate complex oboscure capsule which consists of a liquid chitosan core with an alginate coating around an interphasic membrane is formed by dropwise addition of chitosan solution through a stainless steel cannula into an alginate solution. An optimum capsule formation procedure along with the materials and methods used are described in [6, Pp. 77-78].

Daly shows that the addition of calcium chloride in the chitosan solution increases the membrane structural stability and enhances the uniformity in size and shape of the capsules, because calcium ions can diffuse through the ionic chitosan/alginate and alginate layer and cross-link the alginate acid. The study also shows that a 15 minute reaction time yields the strongest possible capsules in the shortest time.

Algin [7] suggests an addition of glucose (serves as both a plasticizing agent and a dispersing aid) in the alginate solution increases the membrane strength. He shows that the strongest membrane is at sodium alginate to glucose ratio between 0.75 to 1.0% sodium alginate solution and 2.25 to 3.0% glucose.

Eudragit R1

Eudragit R1 is a water insoluble polyacrylate. Such polymer is expected to be more bio-compatible, stronger and less sensitive to process conditions. However, Polyacrylates are more difficult to use than water soluble polymers because of the needs to introduce organic solvents and non-solvents while carefully controlling the temperature, pH, and osmotic pressure. The process for the microencapsulation of cells in Eudragit R1 is used by Sefton [8], [9].

In order to avoid pH or osmotic pressure changes, capsules are prepared by interfacial precipitation. A polymer solution is made to precipitate at the interface between
the solution (dispersed phase) and the immiscible non-solvent for the polymer (continuous phase). The non-solvent is immiscible with the solvent so that precipitation at the interface occurs by extraction of the solvent from the solution phase into the non-solvent phase, leaving the polymer behind to form a shell around the rest of the dispersed phase. For the purpose of microencapsulation, the cell suspension is contained within the polymer solution dispersed phase. The resulting capsules are washed free of non-solvent and solvent and transferred into an aqueous phase.

Capsule curing and recovery are the most difficult and critical stages in this technique. Poorly cured capsules clump together. Oil-rich capsules do not transfer easily into the aqueous phase, and fragile capsules frequently break before the end of the process. Few adjustments can be made to improve the recovery of the cells. Teflon jars are used to prevent capsules from sticking to the jars. Reaction time of more than 20 minutes is used to ensure a complete reaction. A 25% mineral oil is used in the non-solvent bath to enhance extraction. Multiple washes of the capsules with albumin when transferring from the oil-rich phase to the aqueous phase recovers more capsules [9].

The effects of reaction time and alginate concentration on the strength of the capsule membrane are similar to that of ENCAPSEL, in which longer reaction time, higher concentration of polymer solution, or higher polymer to cell ratio will increase membrane strength [9, p.1142]. Selton suggests the presence of small mobile ionic species hinders the formation of Eudragit RL capsule wall, therefore increasing the ionic strength of the Eudragit-RL solution (con. of NaCl) decreases membrane strength [8, p.85].

Cell viability can be measured indirectly by measuring the glucose consumption rate. Eudragit-RL coated alginate immobilized cells consume 14% less glucose than the uncoated ones do. However, Selton suggests that the presence of Eudragit-RL coating does not affect the metabolic requirements of the cells. The lower rate of glucose
consumption is accounted for by a loss of cell viability near the surface of the calcium alginate core because of the strong cell to polymer interactions [10].

Just as ENCAPSEL technique, the presence of Endurant RI membrane has no resistance to insulin, cell nutrients, and small ions diffusion. However, the resistance to diffusion across the membrane has been found to increase exponentially with increasing molecular weight [11]. It is presumed that Endurant coating should effectively restrict the diffusion of much larger molecules such as proteins.

In comparison to ENCAPSEL, capsules coated with Endurant RI are significantly more durable than the ones coated with polylysine, and do not require further covalent stabilization to produce mechanically durable alginate beads [12].

**Polyethylenimine-Heparin Protamine**

This new membrane technique, which prepared from polyethylenimine, with heparin and protamine sulfate added, is proposed by Krystyna Tatakiowicz. The materials for its production are commonly used in medical treatment. Protamine sulfate is added for the neutralization of an over dosage of heparin. Together they create a very strong, non toxic, and hardly dissolvable complex [13]. This technique is a modification of Lim and Sun [14], and is described in [14].

Microcapsules produced using heparin and protamine are resistant to mechanical stresses. Several passes through the syringe, as well as stirring in a magnetic stirrer, cause no breakage of the membranes. It also has been observed that the membrane is permeable to glucose, insulin (M.W. 5,200), but not permeable to albumin (M.W. 69,000) and r globulin (M.W. 156,000) [12].
Encapsulation Apparatus

The first microcapsules were prepared by dispersing a cell suspension and adding that dispersion rapidly to a vigorously stirring mineral oil bath. Capsules produced were very small (≈ 50-100 μm in diameter) and variable in their properties. Capsules were also subjected to very high shear forces. By adoption of coextrusion process, cells are no longer subjected to very high shear rates so more fragile cells may be encapsulated without mechanical damages. Capsule sizes can be controlled by setting the air flow rate, and capsules can be produced with reasonable uniformity. Contacts between the cell suspension and the polymer solvent is minimized through the two-needle assembly so that solvent related cell injury is kept to minimum.

Setton designed a microencapsulation apparatus which forces cell suspension and polymer solution through a double barrel coextrusion nozzle. An air jet, at constant pressure, is fed through a larger concentric tube, moving past the inner needle at constant velocity. As the droplet begins to form, its surface extends into the annular stream of air, and when it reaches a diameter such that the drag force on its surface exceeds the surface forces holding it to the orifice of the needle, it detaches.

An advantage of this technique is that the size and the size distribution of the droplets are independent of the liquid flow rate over a wide range. Thus, droplets can be formed rapidly at the rate of a milliliter in a few seconds. Drop sizes can be controlled by varying the velocity of the gas stream. By inserting a stylus into the center of the inner needle, droplets as small as 40 μm in diameter can be formed [15].

Capsule Rupturing Methods

Two mechanical methods of disrupting microcapsules are currently being used. The first method consists of lysing the microcapsules using a dounce homogenizer. This is
accomplished by first washing the settled capsules with NaCl solution, resuspending the capsules to their initial harvest volume, then disrupting them with a single stroke of the A tolerance plunger [16]. The second method of rupturing the capsules is simply by passing them several times through a narrow pipe or needle. Cell viability following either treatment can be judged by the exclusion of trypan blue dye.

A chemical method for disrupting capsule specificity for NECAPSIL process is done by first washing the capsules three times with 150 mM NaCl and incubating them at 37°C for 10 minutes in a solution containing 4,000 units/ml of Na heparin in 75 mM calcium chloride. After this period, the microcapsules are washed three times with 150 mM saline and then transferred to a solution of 85 mM Na citrate in 75 mM NaCl, incubate the solution at 37°C for 5 minutes. Following this treatment, the capsules should be completely disrupted and the cells are released as a homogeneous suspension [16].

Toxicity of Encapsulation Solutions and Polymers

The analyses of the toxicities of encapsulation solutions and polymers on cells have been done by Dangulas [17]. It seems encapsulation solutions, CHES, calcium chloride, KCl, sodium citrate, and low M.W. of PEl (22,000), do not have any apparent effects on the cell growth. However, direct contact between a high M.W. of PEl (102,000 and 270,000) and cells results in 75% loss in cell viability. The study also shows the M.W. of chitosan and the concentration of alginate do not have a significant effect on cell viability. A table of analysis summary is given in the Appendix A.

Finally, microcapsules with mutants are needed to be differentiated from the others after the growth phase is over. Separation of mutants from non-mutants is based on the detectable differences, such as the difference in the number of cells per capsule or the density of the capsule. Techniques like equilibrium density centrifugation, or velocity
sedimentation, will detect the differences in cell mass and density. These and many other techniques for screening mutant microcapsules are covered in Perlman's patent [13].

**MATERIALS AND METHODS**

**Materials**

A 1.4% (w/v) sodium alginate solution was prepared by dissolving algic acid (sodium salt, *Macrospora Puerpera*, Low viscosity, Sigma Chemical Company, St. Louis, MO) in 1 L of pure water at a slightly elevated temperature to accelerate the dissolving process. The sodium alginate solution was then sterilized by sterile filtration. Sterile filtration was preferred over autoclaving because high temperature might cause depolymerization of the alginate chains [14]. The alginate solution was filtered directly through a 0.20 μm membrane filter (Nalgene Disposable Filterware, Rochester, NY).

The complexing solution, calcium chloride, was prepared by dissolving calcium chloride powder (Calcium Chloride dihydrate, Fisher Scientific Co., Fairlawn, NJ) in 1 L of pure water. The calcium chloride solution was then sterile filtered through a 0.20 μm membrane filter the same way as the alginate solution.

**Encapsulation Apparatus**

The overall microencapsulation apparatus design is shown in Figure 1, and the needle-sheath assembly is shown in detail in Figure 2. The needle assembly was fabricated by fusing a 1 mm ID glass capillary tube to a ground glass stopper. A roughly 3/4 inch needle was cut from a stainless steel 30 G needle (Small Bore Stainless Tubing).
Master Cam using EDM (Electrical Discharge Machine) in the machine shop at RAI. The needle was wrapped with black tape so it would be firmly fitted to the end of the capillary tube. Then epoxy glue was applied to prevent leakage of the solution through the connection. Care must be taken to prevent direct contact between processing solution and epoxy glue because epoxy might be harmful to living cells. The length of the needle assembly from the bottom of the ground stopper, where the air stream entered, to the tip of the needle was 4 inches. A 5 mm ID, 8 mm OD glass tube was made with a female joint on top so that the ground stopper of the needle assembly would rest firmly in the joint. A glass side arm 4 mm ID, 8 mm OD, 5 cm long was fabricated at 45 degree angle to the glass tube immediately below the female joint. The length of the glass tube was adjusted so that the tip of the needle lined up evenly with the glass sheath. Because it was difficult to fuse the capillary tube straight and centered to the ground stopper, manual centering of the needle in the glass sheath was necessary by rotating the needle assembly until a position closest to the center was achieved.

A Teflon compressed air tank was used to provide the air jet. The air stream was passed via Tygon tubing through a filter (Type A415 DX, Beeston Filter Products, Levant, ME) and a rotameter (No. 4, Cole-Parmer Instrument, Great Neck, NY) before the needle-sheath assembly. Alginate solution was pumped through the capillary tube and the needle via Silicone tubing (4 Flex tubing, Size 13, Masterflex, Cole-Parmer Instrument Co., Chicago) using either a 10 or 20 cc Becton Dickinson syringe with 18G 1/2" Precision Glide Needle mounted on a single drive infusion syringe pump (Razel, model A-99, Razel Scientific Instruments, Stamford, CT). The bead size was controlled by adjusting the air flow rate.
Experimental

Calcium alginate beads were produced by turning on the infusion pump and the air flow. Droplets blown off the needle assembly were collected 45 cm underneath the needle in a 1000 ml beaker containing approximately 200 ml of CaCl₂ curing bath. The air flow rate and the alginate solution flow rate were set at 46.87 l/min and 20.66 cc/hr respectively to produce various sizes of droplets. A magnetic stirrer was placed under the curing bath and set at the lowest speed using a small stirring bar (1/2 X 1/8) to provide thorough interaction between calcium ions and alginate. Prior to extrusion, the curing bath was covered with a paper towel, and the needle assembly, the curing bath, the air, and the solution flow rates were adjusted to produce a steady droplet stream.

After extruding for approximately 5 minutes into the curing bath, the beaker was removed and the solution continuously stirred for 10-15 more minutes. The beads were removed from the beaker using a pasteur pipet and placed into a capped 15 ml disposable polypropylene centrifuge tube (VWR Scientific Inc., Philadelphia, PA) after allowing them to settle in the beaker.

The effect of alginate concentration on the shape and the size of beads was studied. Beads were produced using four Na alginate solutions from 0.5 to 2.5% (w/v) at air flow rate 57 l/min, sodium alginate flow rate 60 cc/hr, and 2.5% w/v calcium chloride solution. To study the effect of calcium chloride concentration on the shape of the beads, four calcium chloride solutions from 1 to 4% at 2.5% w/v alginate concentration, 57 l/min air flow, and 60 cc/hr liquid flow rate, were utilized with varying degree of success.

The distance between the needle tip and the calcium chloride solution was varied in the range of 15-45 cm while holding the air flow rate, liquid flow rate, and calcium concentrations constant at 57 l/min, 66 cc/hr, 2.5% w/v, and 4% w/v, respectively to optimize the shape of the beads.
The effect of stirring on the bead shape was also investigated in this project. Beads were produced with various degrees of stirring during extruding. The beads were then cured under stirring at the lowest speed for an additional 10 minutes and settled for 5 minutes before transferred to a polypropylene centrifuge tube. The stirring experiment was performed at an airflow rate 571/min, liquid flow rate 60 cc/hr, 21% alginate solution, 4% calcium chloride solution, and 45 cm between the tip of the needle and the curing bath.

Beads were transferred to a microscope slide using a pasteur pipet, covered with a cover slip, and observed under a microscope (Zeiss, W. Germany) at 100 X magnification. Pictures of the beads were taken with a 35 mm camera mounted on top of the microscope.

The size of the beads was determined by comparing the beads with standard 96 µm beads (Polybead Polystyrene Microspheres, Dia 95.6 micron, SD 4.5 micron, Polysciences Inc., Warrington, PA). The size of a sample was determined by choosing four beads which best represent the sample and averaging their sizes.

Many revisions were made before the current encapsulation apparatus design was finalized. A chromatographic needle (2.5 inches, Curtin Matheson Scientific Inc., Houston, TX) was first used instead of the present needle-capillary assembly. The needle was inserted into a cork which served as the stopper for the glass tube. A few problems arose with this design. First, the cork was too soft to hold the needle in place. Centering of the needle was almost impossible. Second, spherical droplets were not obtained because the needle tip was tapered asymmetrically which caused the droplets to form from the side of the needle. Third, the needle was too short for an uniform air flow to develop at the end of the needle. Longer needles with small diameters were not available from the company.

Chromatographic needles were therefore discarded from the design and stainless steel needles were used instead. A ground glass stopper with one inch, 1 mm ID capillary tube fused at the center was utilized instead of a cork stopper (Figure 3). The stainless steel needle was machined to 6 inches in length and epoxy glued to both the ground stopper and
the capillary tube. The outer glass sheath was made one inch longer than the needle assembly to prevent the exit effect of the air stream. The exit effect was later found not to be an important factor because the shape of the beads produced with the glass tube longer than the needle did not look any differently than the beads with needle aligned evenly with the tube. The needle assembly was firmly attached to the outer glass tube and was centered to a reasonable degree. However, the needle vibrated violently at high air flow rates and the vibrations caused droplets to smear on the side of the glass wall.

To eliminate the vibration of the needle at high air flow rates, the glass capillary tube below the ground stopper was extended to cover all but the last half inch of the needle. The end of the needle was aligned evenly with the bottom of the glass tube in this design. Epoxy glue was applied to both ends of the capillary tube to hold the needle in place. The result was not satisfactory because the whole needle assembly would still vibrate at very high air flow rate (flow rate = 57 L/min). There was another problem arose with this design. Because the viscous nature of N. alginate solution, pushing the solution through a gauge 30 needle of length 6 inches created a very large back pressure that caused the clamp on the syringe pump to slip indefinitely. Therefore, the needle was further reduced from six inches to 3/4 of an inch, and epoxy glued to the end of the capillary tube. The needle vibration problem was eliminated with this design; however, back pressure still build up gradually and caused the infusion pump to slip after a few minutes of processing. An attempt was made to reduce back pressure by reducing the viscosity of sodium alginate solution. Even at one percent alginate a 50% the pump slipped after a short period of time. No solutions has yet been proposed at this point.
RESULTS AND DISCUSSIONS

**Effect of Needle Tip on Bead Formation**

Uniform and spherical beads were produced using the designed apparatus at various operating conditions. However, size and sphericity of the beads were highly dependent on the smoothness of the needle tip. Two needle assemblies, one with the tip manually polished by a metal file after machining to its desired length, and the other needle left untouched after cutting, were constructed and identical tests (various air and liquid flow rates) were performed. Beads produced using both needles were very spherical and uniform at low air flow rates (16-28 l/min) (Fig 4-7). However, at higher air flow rates (40-57 l/min), majority of the gel beads produced with the unpolished needle were elliptically shaped and some even had tails (Fig. 8-10). Beads generated by the polished needle were very consistent in size and shape at any given air and liquid flow rates.

Two considerations were reflected on the design of the encapsulation apparatus: a uniform air flow down the outer glass sheath, especially at the tip of the needle where droplets form, and a needle that produces spherical beads. The sphericity of beads was thought to depend on the uniformity of the shear force produced by the jet air stream. As a droplet begins to form and extends past the annular stream of air, it will reach a diameter such that the drag forces on its surface exceed the surface force holding it to the orifice, and the droplet will detach from the needle tip. If the air stream around the droplet does not have an uniform symmetric velocity profile, the forces on the droplets will be uneven and cause the droplet to be non-spherical. A well-developed plug air flow (turbulent flow) across the needle tip is therefore desired to ensure symmetric shear forces around the droplet. The minimum air flow rate for a turbulent flow (Re > 3,000) to occur with 5 mm
tube diameter is 10.1 mm (Appendix D: Sample Calculation). The lowest air flow rate tested in this project was 16 L/min, well advanced in the turbulent region. A length to diameter ratio of 22 was allowed in the needle sheath design to minimize entrance effects of the air stream and ensure a developed air flow. The needle was also centered to a reasonable degree, so the air flow past the droplet should be uniform.

The shape of the beads was also thought to depend on the smoothness of the needle tip. Based on the observation of the experimental results, the rough needle tip forced liquid droplets to exit the tip asymmetrically. The droplet shape appeared to be independent of the needle tip at low air flow rates. However, many broken beads and irregular shaped beads were observed with the bad needle at high air flow rates. All the results reported below were based on the data obtained from the good needle.

**Effect of Air Flow Rate**

The highest air flow rate possible with the present air regulator was 57 L/min, and therefore air flows above 57 L/min were not tested. The alginate beads produced were in the range of 700–300 μm with air flow rates 16–57 L/min. Alginate beads shrunk when they were dehydrated. For example, a 300 μm bead would shrink down to 200 μm when it is dry. Therefore the diameter of the beads was measured when the beads were still wet on the microscope slide. Bead size at various air and liquid flow rates are shown in Table 1. Bead size decreased with increasing air flow rate at all liquid flow rates tested (Fig. 11-15). The bead size appeared to decrease linearly with increasing air flow rate until it reached 400 μm at 40 L/min air flow rate. After 400 μm, the bead size started leveling off with increasing air flow rates and reached 300 μm at air flow rate 57 L/min.

There were three factors seemed to govern the size of the alginate gel beads, the drag force by the air stream, the needle size, and the viscosity of the liquid extrude. For a constant sodium alginate concentration, the drag forces and the needle size determined the
size of the beads. As one would have expected, the time from exit to detachment of a
droplet decreases as the air speed increases, and therefore, at a constant liquid flow rate,
droplet size decreases with increasing air flow rate. The smallest sphere diameter that can
be produced, however, is not depend on the air flow rate but is limited by the diameter of
the needle. Theoretically, the smallest bead which can be produced should have the same
diameter as the needle orifice. A gauge 30 stainless steel needle, with inner diameter 152
μm and outer diameter 305 μm, was used in this project. Allowing non-ideality of the air
flow at the needle tip where the tip was cut without tapering, droplets with diameters
around 290 μm should be or close to the smallest bead that can be produced with a gauge
30 needle.

**Size Distribution of the Beads**

The size distribution varied with different size beads produced. In general, small
beads were more uniform than the larger ones (Table 1). The standard deviation decreased
tremendously for air flow rates above 4012/min, or bead sizes smaller than 400 μm. This
phenomenon might be explained by the fact that the larger beads dangled longer at the end
of the needle, waiting for the air to shear them off the tip, so large size variation was
possible. For smaller beads, droplets were torn off the tip by large shear forces as soon as
they formed and so smaller size variations occurred.

**Effect of Alginate Flow Rate**

The effect of the alginate flow rate on the bead sizes is shown in Figure 16. After
taking the variance of the samples into account, liquid flow rates did not seem to have an
effect on the size of the beads. Other researchers have also shown that bead size is
independent of liquid flow rates [11, 115]. This phenomenon was expected because the
size of the droplets depends only on the shear force, needle size, and the viscosity of the
extrusion solution, as discussed in the above paragraphs. Increasing liquid flow rate would only increase bead production rate.

One of the objectives of this project was to produce alginate beads at the highest solution flow rate possible. The solution flow rate was determined by multiplying the cross-sectional area of the syringe by the pump infusion rate. Therefore, it is desirable to use the largest possible syringe to give the maximum flow rate. However, to push a viscous material such as sodium alginate solution through a fine gauge 30 needle requires a very large pump head, and the larger the syringe, the bigger the back pressure will be. Currently, the alginate beads were produced using a 20 cc syringe. The clamp on the pump slipped occasionally because of the large back pressure which the pump had to push against. Switching to a larger syringe would cause the pump to slip even more frequently and the extrusion rate would not increase considerably. More work is still needed to reduce the back pressure and to maximize the liquid flow rate.

**Effect of Na Alginate Concentration**

The size of alginate beads decreased dramatically with decreasing sodium alginate concentration. The bead size dropped from 284 μm at 2% w/v alginate, to 220 μm at 1.0, 1.5% w/v alginate, and 165 μm at 0.5% alginate solution (Fig. 17). However, the sphericity and the size uniformity worsened as the concentration decreased (Table 11). Beads produced at 2% alginate and 57 l/min of air flow rate were very uniform and spherical (Fig. 18). Beads produced at 1.5% alginate were still uniform and looked similar to the ones at 2% alginate but had some elliptical and "tadpole-like" beads (Fig. 19). At 1.0% alginate solution, even though the average size of the beads was the same as that of 1.5%, the beads were in general shaped like "tadpole" and variable in sizes. The color of the beads also became lighter under the microscope (Fig. 20). The beads at 0.5% alginate looked worse than all the other ones. The color of the beads was lighter than before. 

percent of the beads were irregularly shaped. These defective beads generally have rounded fronts but “fish tail” like back (Fig. 21).

The viscosity and the surface tension of alginate solution decrease with decreasing concentration. Therefore, one would expect that smaller forces would be required to tear a less viscous droplet from the needle. It was evident from the experiment that the droplet size decreased with less concentrated alginate solutions. However, damages to the beads are also more likely to occur with decreasing surface tension. The impact resulted from droplets hitting the surface of the curing bath at very high velocity generated by high air speed (571/min) might be detrimental to the beads. When the droplet plunged into the calcium chloride solution, the friction force from beads moving through the curing bath overcame the surface tension of the droplets and thus beads were forced into elliptical shapes or “tear drop” like shapes. Some beads were even broken by the overwhelming impact and shear forces. The clear nature of the beads might be the result of loose matrix structure from low concentration of alginate concentrations. One method one might be able to apply to prevent formation of tear drop beads is by increasing the concentration of the calcium chloride (which is described in detail in the next section) so the beads react and harden instantly at the moment they are in contact with calcium chloride.

**Effect of Calcium Chloride**

The concentration of calcium chloride was varied from 4% w/v (0.36 molar) to 1% w/v (0.09 molar), and with the exception of 1% w/v calcium chloride, beads produced under all the other calcium chloride concentrations were beautifully spherical and uniform in size (Fig. 22, 23). The concentration of calcium chloride did not affect the size of the alginate bead but did affect the shape of the beads at calcium concentration below 2%. The average sizes of the beads under these concentrations were identical at 285 μm, but nearly 50 percent of the beads produced at 1% w/v CaCl2 have tails (Fig. 24).
Alginates constitute a family of unbranched binary copolymers of 1-4 linked \( \beta \)-D-mannuronic acid (M) and alpha-L-guluronic acid (G), of widely varying composition and sequence, depending on the organism and tissue they are isolated from. Divalent cation, such as calcium, binds preferentially to G blocks in a highly cooperative manner; the size of the cooperative unit is reported to be more than 20 monomers. Bonds form between calcium ions and the G block, giving rise to junction zones in the gel network (11). The length of the G blocks is, therefore, the main structural feature contributing to gel formation. High calcium ion concentration gradient accelerates the diffusion of calcium ions through the binding sites in the alginate G blocks and shortens the gel formation time. The beads produced at calcium chloride concentration between 2-4% w/v did not have tails probably because calcium ions diffused rapidly through the alginate polymer and bound with the G block as soon as the droplet entered the curing bath, alginate matrix formed before the friction forces have any effects on the droplets. However, at 1% w/v calcium concentration, diffusion of \( \text{Ca}^{2+} \) ions was slow and the alginate droplets were damaged by the friction forces before the beads completely reacted, tails formed and hardened as the beads moved through the solution.

**Effect of Droplet Falling Distance**

The distance between the needle tip and the surface of the calcium chloride solution was varied from 15 to 45 cm to study the effect of falling distance on the formation of the beads. The falling distance did not have any effects on the shape or the size of the beads at distance between 38 and 45 cm (Fig. 25-28), but as the distance above the curing bath decreased the variations in size and in sphericity increased. Clumps of beads along with elliptical beads were observed with distance 30 cm (Fig. 26). More irregular shaped beads were found with distance 15 cm between the needle and the curing bath (Fig. 27). Instead
of tear drop shape, the shape of the beads at this distances did not have a particular pattern: some were squares, some were triangles, and some were ellipsoids.

It is likely that a droplet loses its sphericity at the moment it snaps away from the needle tip. The recoil force causes the whole bead to vibrate as it falls. The shape of the vibrating bead will gradually reach a steady state, hopefully spherical in this particular case. Therefore, a distance between the syringe and the curing bath was provided to allow the sphericity of the droplet to develop before it plunged into the curing solution. From the experimental results, a distance of 38 cm was sufficient for droplets to establish sphericity. With distances less than 30 cm, the droplets not only were not spherical, but their shapes also indicated physical damages resulting from collision with other beads. At the distance 115 cm where the exit of the air stream from the needle assembly was just above the curing bath, the direct blowing of the air jet caused a turbulence near the surface of the solution. Droplets collided with each other in the midst of turbulence, some formed clusters, and some were damaged.

Effect of Stirring of the Solution

Various stirring speeds (no stirring, low, and high speed) were applied during the extrusion of alginate beads to study the effect of stirring on the formation of the beads. Surprisingly, no noticeable differences on the shape of the beads were detected with all three stirring speeds, except at the highest stirring speed a small number of bead clusters were found (Fig. 18, 28, 29). One would expect that vigorous stirring would cause the beads to form tails due to the increasing drag force. It was not so in this case; the calcium ion concentration and the viscosity of the droplet used were high enough (4% w/v and 2% respectively) that droplets reacted and hardened before they were affected by the shear of the stirring. The clumps of beads observed at the highest stirring speed were probably the result of bead collision caused by the stirring. Overall, at 4% w/v calcium chloride and 2%
alginate concentration, stirring of the solution during extrusion did not affect the uniformity and the sphericity of the beads.

CONCLUSIONS

In this project, we have successfully designed an alginate bead generator apparatus and optimized various operating conditions that produced uniform and spherical beads at desired sizes. The smallest beads produced with good sphericity and uniformity were of the samples were around 200 μm, and they were produced under these operating conditions: 2.0% w/v sodium alginate solution; 2.4% w/v calcium chloride solution; 5°C 1/L min air flow rate; 60-66 cc/hr alginate flow rate; 3-45 cm distance between needle tip and calcium chloride solution; and low speed or no stirring of the curing bath during extrusion.

Future investigations include: 1) finding a needle cutting method that will consistently provide smooth needle tips; 2) increasing the present maximum liquid flow rate (66 cc/hr) by using a larger syringe or pump; 3) developing the PLL membrane formation technique; 4) of course, starting the encapsulation of yeast cells.
BIBLIOGRAPHY


16. Allan P. Jarvis, Jr. and Therese A. Grima, Production of Biologicals from Microencapsulated Living Cells, BioTechniques.


Table 1: The Effects of Liquid and Air Flow Rates on the Bead Sizes

Na alginate concentration: 3% w/v
Calcium Chloride concentration: 4% w/v
Falling distance: 48 cm
Stirring speed: low (1/2 X 1/8)

<table>
<thead>
<tr>
<th>Liquid flow rate (cc/hr)</th>
<th>Air flow rate (L/min)</th>
<th>Ave. Bead Size (μm)</th>
<th>Standard Deviation (μm)</th>
<th>(S.D. x Mean x 100)</th>
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Table II: The Effect of Na Silicate Concentration on Bead Size

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<tr>
<th>Silicate concentration (wt%)</th>
<th>Avg. Bead Size (μm)</th>
<th>Uniformity (mm)</th>
<th>Sphericity (%)</th>
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<td>0.2</td>
<td>0.2</td>
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<td>1</td>
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<td>1.5</td>
<td>50</td>
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<td>0.9</td>
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<tr>
<td>2</td>
<td>66</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*These are based on eye inspection of a sample under a microscope.*
Figure 1: Microencapsulation apparatus. Na alginate solution is pumped through needle sheath assembly. Droplets at end of needle are blown off by coaxial air stream and collected in CaCl₂ curing bath.
Needle Assembly

Ground Glass Stopper

Top View

Fused Capillary Tube to Ground Glass Stopper

Extended Capillary Tube

3/4" G 30 Needle

Female Joint

Outer Glass Sheath

ID 5 mm

Figure 2. Detail illustration of needle sheath assembly. Na alginate solution are pumped through capillary tube and needle.
Figure 3. Schematic illustration of old encapsulation design. Capillary tube only extended one inch below the glass stopper.
Figure 6: Beads produced by the polished needle at 16 L/min air flow, 4" x 4" in liquid flow, 2% w/v alginate, 4% w/v calcium chloride, low speed stirring, and 48 h in the distance between needle and stirring bath. Beads were in general very uniform and spherical. (magnification 100X)
Figure 7: Beads produced by the polished needle at 28 L/min air flow, 47 cc/hr liquid flow, 2% w/v alginate, 4% w/v calcium chloride, low speed stirring, and 45 cm the distance between needle and curing bath. Beads were in general very uniform and spherical. (magnification 100X)
Figure 13: Bead Size vs. Air Flow Rate  
(at Liquid Flow Rate 47 cc/hr)

Figure 14: Bead Size vs. Air Flow Rate  
(at Liquid Flow Rate 60 cc/hr)
Figure 15: Bead Size vs. Air Flow Rate
(at Liquid Flow Rate 66 cc/hr)
Figure 16: The Effect of Liquid Flow Rate on Bead Size

- ○ Bead Size (μm) [20 cc/hr]
- □ Bead Size (μm) [34 cc/hr]
- □ Bead Size (μm) [47 cc/hr]
- ▲ Bead Size (μm) [50 cc/hr]
- + Bead Size (μm) [56 cc/hr]
Figure 17: The Effect of Alginate Concentration on the Bead Size
Figure 18: Beads produced by the polished needle at 57 l/min air flow, 60 cc/hr liquid flow, 2% w/v alginate, 4% w/v calcium chloride, low speed stirring, and 45 cm the distance between needle and curing bath. Beads were very spherical and uniform in size. (magnification 100X)
Figure 7 Beads produced by the poloset needle at V1. At 0.5 m/min. at 54 in.
liquid flow, 0.05 mm at 0.05 mm glass, 0.05 mm open to air, 1000 rpm. The distance between the beads was approximately 0.5 mm. Majority of beads were irregular shaped or broken. Beads were almost transparent. Magnification: 100X.
Figure 3. Beads produced by the polished needle at 5.7 atm air flow, 50 cc/min liquid flow, 20% w/w alginate, 0.0% w/w calcium chloride, low speed stirring, and 45 cm the distance between needle and cooling bath. Beads were uniform and spherical, no different than the ones at 4.5% w/w calcium chloride. Magnification 100X.
Figure 28. Beads produced by the polished needle at 30 mm air flow, 10 cc/min liquid flow, 20% w/v alginate, 4.0% w/v calcium chloride, low-speed stirring, and 38 cm the distance between needle and culture bath. Beads were uniform and spherical, no different than the ones at 38 cm tailing distance. Magnification 100X.
Figure 7. Beads produced by the polished needle. 10% containing 45% liquid flow, 20% air argon, 10% water, 45% microtextured aero spaced stirs, and 20% in the distance between needles. Of the 10% 80% of the beads were encapsulated and 20% of the other half were formed. 400 strains were carried out [105].
APPENDIX A: Toxicity of Solutions on Cells

Table 1. Toxicity effect of PLL and encapsulation solutions on insect cells.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Percentage of Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL Solution (0.075 w/v)</td>
<td></td>
</tr>
<tr>
<td>MW 12,000</td>
<td>100</td>
</tr>
<tr>
<td>MW 10,000</td>
<td>100</td>
</tr>
<tr>
<td>MW 8,000</td>
<td>100</td>
</tr>
<tr>
<td>Citrate</td>
<td>100</td>
</tr>
<tr>
<td>CHES</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>100</td>
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</table>

Table 2. Toxicity effect of various molecular weight Protan Chitosan with and without contact time 1 minute.

<table>
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<th>Type of Chitosan</th>
<th>Approximate MW</th>
<th>Viable Cells</th>
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</thead>
<tbody>
<tr>
<td>Sea Cure 1 w/v</td>
<td>408,000</td>
<td>100</td>
</tr>
<tr>
<td>Protan 1 w/v</td>
<td>968,000</td>
<td>80.4 ± 0.6</td>
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</table>

Table 3. The effect of alginate on cell viability with varying alginate cell contact time and alginate concentration.

<table>
<thead>
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<th>Alginate Concentration</th>
<th>Contact Time (min)</th>
<th>% Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4% (w/v)</td>
<td>1</td>
<td>80 ± 10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>78 ± 22</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>96 ± 7</td>
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<tr>
<td>0.7% (w/v)</td>
<td>1</td>
<td>98 ± 8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>78 ± 26</td>
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<td></td>
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<td>KCl control</td>
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<td>5</td>
<td>100</td>
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<td></td>
<td>20</td>
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</table>
APPENDIX B: Technical Details on Making Alginate Beads

I. Concerning the Needle:

We only use gauge 30 needles, so make sure we have enough of them on hand. Order them a few weeks in advance to allow delivery and processing. If time permits, have the machine shop cut the needle to the desired length (about one inch) using EDM. It usually takes two to three weeks for the machine shop to do it (they say the EDM is only free on Fridays). The EDM machine supposed to prevent impinging of the needle at the cut point, but sometime it does not do a good job. So always check the needle tips before using them. If you are in a hurry to make beads and one end of your needle is in good condition (even and smooth that can be used as the outer needle tip to produce spherical droplets), the glass shop at Noyes Lab has a couplerendum saw that can cut the needle too. Sometimes the couplerendum saw will impinge the needle a little bit, but you can sand it smooth with a metal file.

II. Making the Needle Assembly:

After the needle is cut to the desired length, wrap the needle with enough Teflon tape (roughly 3/4 inch) so that the needle would fit tightly in capillary tube at the end of the needle-sheath assembly. Mix equal amount of epoxy resin and harder, and apply to the capillary tube and the Teflon tape. There is no specific way of how to glue the needle, do it any ways you want as long as the needle assembly does not leak. Don't put too much glue otherwise it might impede the air flow. Let the glue air dry for at least 24 hours.

III. Cleaning and Unplugging the needle:

Most of the time Na alginate solution will dry out and clog up the needle if it's been sitting idle in the needle for a while. To unplug the needle simply immerse the needle assembly in a 60°C heat water bath for 10 minutes. Then pass the 60°C hot water through
the needle. The needle should be unplugged without any difficulties. Warning: the epoxy glue cannot withstand heat (unless the epoxy glue you used is water and temperature resistant) so you might have to re-glue the needle after immersing in the hot water. One way to prevent clogging of the needle is to always pass hot water through the needle right after each bead-making process to make sure that no alginate remains in the needle. This way you don't have to waste time unplugging and re-gluing the needle assembly.

IV. Solution Preparation:

A. Na alginate solution:

Dissolve appropriate amount of low viscosity alginate acid in pure water by gently heating on the hot plate and constant stirring of the solution. Filter the dissolved solution using a Whatman size 2 filter paper (Whatman International Ltd.) first. Then sterile-filter the filtrate through a 0.20 μm pore 150 ml Nalgene disposable filter. Each filter will filter about 30 ml of solution before it got clogged up.

B. CaCl₂ solution:

Dissolve appropriate amount of CaCl₂ dihydrate in pure water. CaCl₂ dissolves easily in water, so no heating is needed. Sterile-filter the solution using a 0.20 micron 250 ml Nalgene disposable filter. CaCl₂ solution is pretty pure so one filter is all you need to filter 2 L of solution.

V. Calibration of the Infusion Pump:

Copies of the calibration charts for 5, 10, and 20 cc syringe are enclosed in Appendix C. All the pump rates recorded in this project were based on these charts.
VI. Concerning the Study of Beads:

After the beads react with CaCl₂ solution for at least 15 minutes, transfer the beads and the CaCl₂ solution from the beaker to a polypropylene centrifuge tube using a pasteur pipet. To observe the beads under a microscope, draw a sample using a pasteur pipet from a well-shaken centrifuge tube and slowly place on a microscope slide. Tilt the slide to drain the liquid from the slide as you dispense the sample onto the slide. The beads will stick to the slide automatically. Place a cover slip on the beads. If the beads break that means the beads are not cured yet. To take pictures of beads, make sure the beads of choice are inside the four brackets when viewing from the microscope. Turn on the camera, check to see if there is film in it, and take pictures using the remote control button. For details about the camera, check the camera manual. Kodak TMAX 400 black and white films are good for this kind of picture, and they can be purchased either at the Consolidated Storage Room in Noyes Lab or at the University Photographic Service center on Wright Street. The films can be also developed and printed by the Photographic Service and they can be charged to the account. I found later on that regular Kodak 100 color film is good too and they take less time for the photographic shops to develop.
APPENDIX C: Pump Calibration

5 cc Multifit Syringe:

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20 cc Multifit Syringe:

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RAZEL Model A-99 Pump Calibration of Flow Rate for 5 cc Multifit Syringe
RAZ11 Model A-99 Calibration of flow rate for 10cc Multifit syringe
RAZLI Model A 90 Pump Calibration of Flow Rate for 20 cc Multifit Syringe
1. Air Properties at 1 ATM, 30°C

- Density: 1.29 kg/m³
- Viscosity: 1.85 * 10⁻⁴ kg/ms

Cross-sectional Area of the tube: 
\[ \left( \frac{d}{2} \right)^2 \pi = 0.005 \text{ m}^2 \]

\[ d = \text{diameter of the tube} \]
\[ u = \text{velocity of the air} \]
\[ \rho = \text{density} \]
\[ \mu = \text{viscosity} \]

Reynolds number, \( \text{Re} = \frac{d \cdot u \cdot \rho}{\mu} \)

- At \( \text{Re} = 2100 \), \( u = \frac{\text{Re} \cdot \mu}{d \cdot \rho} = 7.5 \text{ m/sec} \) \( \Rightarrow Q = uA = 8.8 \text{ L/min} \)

- At \( \text{Re} = 3000 \), \( u = \frac{\text{Re} \cdot \mu}{d \cdot \rho} = 8.6 \text{ m/sec} \) \( \Rightarrow Q = uA = 10.1 \text{ L/min} \)

Therefore, theoretically:

- \( Q < 8.8 \text{ L/min} \) Laminar flow
- \( 8.8 < Q < 10.1 \) Transition flow
- \( Q > 10.1 \text{ L/min} \) Turbulent flow

The lowest air flow rate used in this project was 16 L/min, clearly it is in the turbulent flow region.
II. Bead Size Calculation:

The reported bead size of a sample is calculated by averaging the sizes of four beads that best represent the sample.

The standard deviation of the sample \( \sigma \) is defined as

\[
\sigma = \sqrt{\frac{\sum x^2 - (\sum x)^2}{n-1}}
\]

where \( n = 4 \) and \( x \) = the size of each bead

For example:

A sample of beads with sizes: 580 \( \mu \)m
590
780
595

The SD of the sample is 71 \( \mu \)m