

# Journal of Visualized Experiments

## Fabrication of amyloid- $\beta$ -secreting alginate microbeads for use in modelling Alzheimer's disease

--Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE59597R1
<b>Full Title:</b>	Fabrication of amyloid- $\beta$ -secreting alginate microbeads for use in modelling Alzheimer's disease
<b>Keywords:</b>	Alzheimer's disease; amyloid; A $\beta$ ; hydrogels; alginate; encapsulation; 3D in vitro models; controlled release
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<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
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**TITLE:**

Fabrication of Amyloid- $\beta$ -Secreting Alginate Microbeads for Use in Modeling Alzheimer's Disease

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**KEYWORDS:**

Alzheimer's disease, amyloid, A $\beta$ , hydrogels, alginate, encapsulation, 3D in vitro models, controlled release

**SUMMARY:**

This protocol illustrates a cell encapsulation method by rapid physical gelation of alginate to immobilize cells. Obtained microbeads allow controlled and sustained secretion of amyloid- $\beta$  over time and can be used to study the effects of secreted amyloid- $\beta$  in in vitro and in vivo models.

**ABSTRACT:**

According to the amyloid cascade hypothesis, the earliest trigger in the development of Alzheimer's disease (AD) is the accumulation of toxic amyloid- $\beta$  (A $\beta$ ) fragments, eventually leading to the classical features of the disease: amyloid plaques, neurofibrillary tangles and synaptic and neuronal loss. The lack of relevant non-transgenic preclinical models reflective of disease progression is one of the main factors hindering the discovery of effective drug treatments. To this end, we have developed a protocol for the fabrication of alginate microbeads containing amyloid-secreting cells useful for the study of the effects of chronic A $\beta$  production.

Chinese hamster ovary cells previously transfected with a human APP gene, secreting A $\beta$  (i.e., 7PA2 cells), were used in this study. A three-dimensional (3D) in vitro model for the sustained release of A $\beta$  was fabricated by encapsulation of 7PA2 cells in alginate. The process was optimized to target a bead diameter of 500–600  $\mu$ m for further in vivo studies. Optimization of 7PA2 cell encapsulation in alginate was performed altering fabrication parameters, e.g., alginate

concentration, gel flow rate, electrostatic potential, head vibration frequency, gelling solution. Levels of secreted A $\beta$  were analyzed over time and compared between alginate beads and standard cell culture methods (up to 96 h).

A concentration of  $1.5 \times 10^6$  7PA2 cells/mL and an alginate concentration of 2% (w/v) buffered with HEPES and subsequent gelation in 0.5 M calcium chloride for 5 min were found to fabricate the most stable microbeads. Fabricated microbeads were 1) of uniform size, 2) with an average diameter of 550  $\mu$ m, 3) containing about 100–150 cells per microbead and 4) able to secrete A $\beta$ .

In conclusion, our optimized method for the production of stable alginate microbeads containing amyloid-producing 7PA2 cells might enable the modeling of important aspects of AD both in vitro and in vivo.

## INTRODUCTION:

Modeling neurodegenerative disease is challenging due to the complex and intricate nature of the brain. In Alzheimer's disease (AD), the progressive loss of synaptic function and death of neurons is believed to be a downstream effect of sustained overproduction and accumulation of amyloid beta (A $\beta$ ) peptides following abnormal processing of the amyloid precursor protein (APP) according to the amyloid cascade hypothesis<sup>1</sup>.

In order to understand the mechanisms of this amyloid-induced pathology and to aid in the identification of novel treatment targets, scientists have developed various in vivo preclinical models. One category of models utilizes a bolus injection of a synthetic A $\beta$  peptide into the rat brain<sup>2–4</sup>. The main limitation of such models is that they rely on a single-point or repeated treatments with A $\beta$  peptides in high concentrations deposited all in one go. This is inconsistent with the chronic, sustained nature of the release of A $\beta$  in disease<sup>5</sup>. Another category of in vivo models is transgenic animal models expressing one or more genetic mutations linked with the familial variations of the disease<sup>6–10</sup>. However, since familial AD only accounts for fewer than 5% of all Alzheimer's cases<sup>11</sup>, the relevance of these models in translating to sporadic AD in humans is questionable<sup>12</sup>. Another drawback of the transgenic approach is the accelerated A $\beta$  formation from birth, which translates into deficits in cognitive function and pathological changes too quickly and aggressively to resemble disease progression in sporadic AD in patients<sup>12</sup>. For example, the 5x FAD model produces plaques in as little as 1.5 months<sup>13</sup>.

Interestingly, both these categories result in changes in cognitive function of relevance to AD research<sup>2–6</sup>, and sometimes they are accompanied by the appearance of pathological hallmarks of the disease such as amyloid plaques<sup>6,8</sup>, tau phosphorylation<sup>6,7</sup> and/or synaptic and neuronal loss<sup>7,9,14</sup>. But while these types of models may give us an insight into the effects of high levels of amyloid in the brain, which are often associated with later stages of AD, they fail to reflect the earlier changes exhibited in response to the chronic and sustained exposure to the A $\beta$  peptide<sup>12</sup>, such as altered expression of synaptic markers<sup>15</sup> and components in the extracellular matrix<sup>16</sup>. Therefore, there still remains a need for creating a chronic model that more accurately illustrates the effects of sustained A $\beta$  secretion on in vivo cognition and illustrates changes in pathology.

To this end, we have developed a system which allows the constant, sustained secretion of A $\beta$  in a controlled manner by immobilizing amyloid-secreting cells within hydrogel microbeads, which can be subsequently implanted within the adult rat brain to model aspects of sporadic AD.

Alginate was the selected biomaterial as it is biocompatible and does not induce any adverse responses when implanted in vivo<sup>17</sup>. Cell encapsulation in alginate hydrogels has been well established over the last four decades. The first example of its translation to the clinic was reported for the treatment of type 1 diabetes mellitus<sup>17</sup>. The earliest report of successful encapsulation of islets of Langerhans dates back to 1980. Transplantation of microbeads containing insulin-secreting cells revolutionized treatment options for diabetic patients as it restored pancreatic function, eliminating the need for insulin injection therapy<sup>18</sup>. These works report on how cell encapsulation can protect them from external stresses, whether mechanical or chemical. In fact, alginate beads act as a barrier and isolate cells from the surrounding environment preserving their phenotype, whilst allowing sufficient access to the surrounding media for nutrients and clearance of cellular byproducts<sup>19</sup>. Moreover, the use of alginate allows matching of mechanical properties of soft tissue<sup>20</sup>. Alginate hydrogels can be tuned to have a stiffness of 1–30 kPa, by simply varying alginate concentration and cross-linking density<sup>20,21</sup>. This is an essential aspect, not only to maintain the phenotypic expression of encapsulated cells in vitro, but also to avoid any inflammatory effects after engraftment in vivo<sup>22</sup>.

In this protocol, 7PA2 cells—a Chinese hamster ovary cell line that is stably transfected with a human APP V717F mutated gene<sup>23</sup>—are used. These cells continuously produce catalytic products of APP, including A $\beta$ <sub>1-42</sub><sup>24,25</sup>, and have been used to generate A $\beta$  as an alternative to synthetic production in preclinical, acute in vivo studies<sup>26</sup>. We describe a fabrication method for immobilizing 7PA2 cells within 'soft' alginate microbeads, designed to allow the sustained secretion of biomolecules. As a proof of concept, we report on the release of the A $\beta$ <sub>1-42</sub> peptide over time. The alginate that is used is a low-viscosity alginate with a molecular weight of 120,000–190,000 g/mol and a mannuronic to guluronic ratio of 1.56 (M/G).

In further studies, these microbeads can be safely transplanted within regions of the rat brain of relevance to AD (e.g., the hippocampus) to study the effects of chronic A $\beta$  secretion on behavior in vivo and pathology ex vivo. In addition, this system can be used to study the effects of chronic A $\beta$  release in in vitro and ex vivo applications. For instance, 7PA2-containing alginate microbeads can be co-cultured in vitro with neuronal or astrocytic cultures to assess the effects of chronic A $\beta$  exposure on cellular mechanisms associated with AD. Furthermore, this method can be used to examine the relationship between chronic A $\beta$  production and long-term potentiation in ex vivo electrophysiology.

The highlight of this protocol is the modularity and flexibility of the fabrication method, which enables the fabrication of alginate beads with a target dimension by fine tuning fabrication parameters. Depending on the application, the protocol can be adjusted to obtain bespoke targets with regards to microbead size, density of the encapsulated cells, and microbead stiffness. This protocol can be used for the encapsulation of a variety of cell types, developing more relevant three-dimensional (3D) in vitro models to study different pathologies. We recently

reported on how alginate-encapsulated cells can be used to model early stages of cancer progression<sup>20</sup>.

The concept of the encapsulation process is based on the extrusion of a laminar jet of cells suspended in alginate solution through a nozzle. A vibrating head disrupts the jet with a controlled frequency resulting in equally sized alginate-based droplets. An external electric field allows separation of the formed alginate-based droplets, which upon contact with a solution enriched in divalent ions, such as calcium ions, can quickly cross-link, preserving their spherical shape. Incubation in the gelation solution allows formation of spherical microbeads containing cells within a homogeneous physical hydrogel<sup>27</sup>. Target size of microbeads and alginate hydrogels allows nutrients and oxygen exchange with cell culture media for long periods of time (weeks). **Figure 1A** show a schematic representation of the encapsulation apparatus used (**Figure 1B**).

[Insert Figure 1]

Microbead size can be altered depending on the intended use. In order to control the size of the microbead, the various parameters outlined in **Figure 1A** and in the protocol are adjusted accordingly. The internal diameter of the nozzle used has a substantial impact on the size of the droplets; further adjusting encapsulation parameters, namely extrusion speed, vibration frequency and voltage, is key to achieving a consistent size distribution. **Table 1** outlines how the different parameters can alter the size of microbeads achieved with this system.

[Insert Table 1 here]

## **PROTOCOL:**

### **1. Preparations**

1.1. Prepare 100 mL of ~4% (w/v) alginate stock solution.

1.1.1. Weigh 4.4 g of alginate sodium salt and add the dry powder to 100 mL of HEPES-buffered saline (HBS, 20 mM HEPES (0.477 g) with 150 mM NaCl (0.877 g) in deionized water allowing hydration.

1.1.2. Use a magnetic stirrer at ~500 revolutions per minute (rpm) and heat to up to 50 °C to allow complete alginate hydration.

NOTE: The alginate may need one or two hours to be completely hydrated. To save on time, alginate solution can be made up a day in advance of the encapsulation protocol and stored at 4 °C until the next day. When alginate has been refrigerated, heat gently to 37 °C using a water bath or a hot plate and agitate with a magnetic stirrer immediately before use.

1.1.3. Sterile filter the alginate solution using a 0.22  $\mu\text{m}$  pore polyethersulfone (PES) filter before use. Filtering is recommended at 37  $^{\circ}\text{C}$  to allow easy flow of alginate. The viscosity will increase if the temperature is allowed to drop.

1.2. Prepare 1000 mL of 0.5 M  $\text{CaCl}_2$ .

1.2.1. Weigh 55.49 g of anhydrous  $\text{CaCl}_2$  and dissolve in 1000 mL of HBS (20 mM HEPES and 150 mM NaCl in 1000 mL of deionized water).

1.2.2. Sterile filter using a 0.22  $\mu\text{m}$  pore PES filter.

1.3. Prepare 100 mL of dissolution mix.

1.3.1. Prepare a 100 mM HEPES (23.83 g) and 500 mM trisodium citrate dihydrate (147.05 g) solution in phosphate-buffered saline.

1.3.2. Adjust to pH 7.4 using NaOH or HCl.

## **2. Setting up the encapsulation system**

2.1. Clean the laminar flow hood.

2.1.1. Sterilize the laminar flow hood containing the encapsulator using UV exposure followed by spraying with 70% v/v ethanol solution.

2.1.2. Flush the encapsulator system with 10 mL of 70% v/v ethanol solution followed by 10 mL of sterile deionized water.

2.1.3. Attach the 300  $\mu\text{m}$  nozzle and repeat step 2.1.2.

2.1.4. Spray the bottle containing the filtered alginate solution, the bottle containing filtered calcium chloride solution and any tools, equipment and empty culture plates that will be used with 70% v/v ethanol solution and place them into the laminar flow hood. Prior to use, switch on a UV light again for 30 min to thoroughly sterilize.

2.1.5. Prepare a waste beaker filled with a biological grade disinfectant solution and place the beaker into the hood.

2.1.6. Fill a beaker with 100 mL of sterile  $\text{CaCl}_2$  (aqueous) solution and add in a magnetic stirrer. Set the stirring speed to 100 rpm. Place the beaker on a magnetic platform at a height of 18 cm from the tip of the nozzle. This beaker will be used to collect alginate beads and allow their gelation.

## **2.2. Prepare cells for encapsulation.**

2.2.1. Remove cells from the incubator and detach from the near-confluent flask using 0.25% trypsin-EDTA solution and incubate at 37 °C for 5–10 min.

2.2.2. Isolate a sample for estimation of cell density and then centrifuge the remaining cells at 1000 rpm for 5 min to obtain a cell pellet.

2.2.3. Resuspend the pellet in HBS (20 mM HEPES and 150 mM NaCl) to double the final desired cell concentration (e.g., for a final concentration of  $1.5 \times 10^6$  cells/mL, resuspend the cells in HBS to achieve a concentration of  $3 \times 10^6$  cells/mL).

2.2.4. In a 50 mL centrifuge tube, mix the cell suspension in a 1:1 ratio with ~4% (w/v) alginate solution to obtain a final suspension containing the desired cell concentration (e.g.,  $1.5 \times 10^6$  cells/mL) in a ~2% (w/v) alginate solution.

2.3. Set the encapsulation parameters.

2.3.1. Set the speed of the encapsulator machine to the maximum extrusion speed (8.9 mL/min), the voltage to 1.0 kV and the frequency to 5,500 Hz.

NOTE: These parameters were previously optimized to obtain microbeads of 550  $\mu$ m in diameter.

### 3. Fabrication

3.1. Fabricate the microbeads.

3.1.1. In a 20 mL syringe, load 5 mL of the cell-alginate suspension and attach a syringe to the encapsulator.

3.1.2. Start the encapsulator by activating the flow which will push the cell-alginate suspension through the feeder. A stream of droplets will be extruded through the nozzle.

3.1.3. Collect the first 1 mL in the waste beaker to void the initial non-uniform stream.

3.1.4. Continue to run the remaining 4 mL allowing the droplets to fall into the  $\text{CaCl}_2$  gelation bath. Each milliliter can be run separately (but successively) and collected in four different gelation baths if required.

NOTE: Upon contact with the gelation bath, the alginate in the droplets will instantly cross-link with the calcium ions in the gelation bath and form spherical microbeads.

3.1.5. After one minute, remove the gelation beaker from the magnetic platform and allow the microbeads to sit for a further 4 min without agitation (time necessary to allow complete gelation across the microbeads at room temperature).

### 3.2. Retrieve microbeads.

3.2.1. Remove any large alginate debris or artefacts using a pair of sterile tweezers, and then use a sterile plastic pipette to transfer to a 74  $\mu\text{m}$  mesh filter to retrieve the microbeads from the gelation bath.

3.2.2. Transfer the microbeads into a centrifuge tube using the appropriate culture medium and allow to equilibrate for 5 min in the cell culture medium.

3.2.3. Transfer to a flask, plate or Petri dish for incubation and further experiments.

NOTE: Gelation bath should not be reused.

## 4. Testing and use of microbeads

### 4.1. Test microbead quality.

4.1.1. To assess the cell viability (or other biological readouts) after encapsulation and culture, gently disrupt the microbeads to release the encapsulated cells using the dissolution mix.

NOTE: The required volume of dissolution mix is dependent on the number of microbeads used per test. A suggested ratio is 4 mL of dissolution mix to every 1 mL of encapsulated cells. This step only needs to be performed on a single sample from each microbead population.

4.1.2. Incubate the cells in a cell culture incubator supplemented with 5%  $\text{CO}_2$  at 37  $^\circ\text{C}$  for 10 min.

4.1.3. Estimate cell viability for cells now in solution as usual by staining cells with trypan blue and using a hemocytometer chamber.

NOTE: If using automated cell counters for estimations of cell viability, care should be taken to avoid alginate artefacts interfering with the counts. In those cases, manual cell counting is advised.

4.1.4. To assess microbead stability, measure the average diameter for a sample from each microbead population over a time course using a microscope and imaging software. Dramatic subsequent variations in diameter can be indicative of alginate degradation.

### 4.2. Use microbeads for detection of secreted $\text{A}\beta$ .

4.2.1. Sample cell culture media from 7PA2 cells cultured in standard conditions (2D) at 24 h intervals and store at -20  $^\circ\text{C}$  for further analysis.



4.2.2. Sample cell culture media from encapsulated 7PA2 cells cultured in standard conditions (3D) at 24 h intervals and store at -20 °C for further analysis.

4.2.3. Detect secretion of A $\beta$  from 7PA2 cells into collected conditioned media by enzyme-linked immunosorbent assay (ELISA) (**Figure 4**).

4.3. Use the microbeads for further study in relevant applications.

NOTE: Encapsulated 7PA2 cells can be used to assess the effects of sustained A $\beta$  secretion in any in vitro or in vivo model.

4.3.1. For microbead engraftment within the rat hippocampus in ex vivo brains, generate 1 mm thick coronal sections of the rat brain and use surgical tools to insert microbeads into the desired area (**Figure 5**).

4.3.2. Encapsulate different cell types in alginate microbeads following the described protocols to assess the sustained release of the biomolecules of interest. Relevant in vitro and in vivo systems can be further modeled.

4.3.3. Detect the expression of membrane-bound markers of encapsulated cells using flow cytometry and/or immunofluorescence.

NOTE: An example of the use of a similar method for the fabrication of microbeads modeling early stages of tumor mass growth and biomarker expression is described by Rios et al.<sup>20</sup>.

## REPRESENTATIVE RESULTS:

### **7PA2 cells are successfully encapsulated in alginate microbeads**

After preparation, uniform and spherical alginate microbeads are successfully generated using this protocol. The images in **Figure 2A** below showcase an example of how altering one of the parameters (i.e., voltage) changes the flow and dispersion of the alginate droplets stream. **Figure 2B** shows an example of obtained alginate beads immediately after the gelation process.

[Insert Figure 2 here]

The described protocol is flexible and allows the fabrication of different sizes of alginate-based microbeads, varying in composition according to the application. In **Figure 2C**, we report the influence of alginate flow rate, voltage and frequency on microbead size using a 2% (w/v) alginate solution in HEPES (pH 7.2) extruded through a 300  $\mu$ m nozzle.

As the scope of this study was to produce microbeads that can be embedded within the rat brain, we adjusted the fabrication parameters to obtain an average microbead diameter in the range of 500–600  $\mu$ m. For quality control purposes, the method was optimized to have minimal variability across the population of fabricated beads (i.e., narrow size distribution). Please note

that (1) produced beads can be injected in the rat brain using a Hamilton syringe equipped with a 20G needle; and (2) one hemisphere of rat brain can host up to two or three beads of this size.

After optimization, encapsulating 7PA2 cells at a concentration of  $1.5 \times 10^6$  cells/mL suspended in a 2% (w/v) alginate solution buffered with HEPES and dropped in a 0.5 M calcium chloride gelation bath yielded the desired microbeads. **Figure 3A** below shows encapsulated 7PA2 cells evenly distributed in microbeads after one day in standard cell culture conditions. 7PA2 cell proliferation was tested using an MTS assay as per manufacturer's protocol. There was no significant difference between the behavior of 7PA2 cells grown with or without alginate over a seven-day period (**Figure 3B**). When incubating encapsulated cells in standard culture conditions, cells are expected to continue to proliferate over the duration of the experiment, and small degrees of cell escape can be expected as a result, as reported in other works<sup>28</sup>. The effects of this can be mitigated by encapsulating at a smaller cell density or reducing the serum concentration in which microbeads are incubated.

[Insert Figure 3]

#### **Alginate microbeads encapsulating 7PA2 cells are stable over time**

To investigate the size and shape of obtained alginate microbeads, microscope analysis was performed after fabrication and gelation. The average diameter for the microbeads obtained using the selected protocol is  $550 \pm 2 \mu\text{m}$ .

Theoretically, the number of cells expected in a  $550 \mu\text{m}$ -diameter microbead can be calculated as follows:

$$V = \frac{4}{3} \pi r^3$$

where  $V$  = volume and  $r$  = radius. The volume of a single microbead is  $V = 8.8 \times 10^{-5} \text{ mL}$ ; therefore, the number of cells per microbead at time of encapsulation =  $(1.5 \times 10^6) \times (8.8 \times 10^{-5}) \approx 130$  cells.

Experimentally, we counted the number of encapsulated cells immediately after fabrication. Alginate beads were gently disrupted using the dissolution mix and cells were stained with trypan blue solution. Viability estimation and counting of cells was performed using a hemocytometer; obtained results showed an average of  $116 \pm 17$  live cells per microbead ( $n = 5$ , data not reported). As expected, a minor difference between theoretical and experimental cell count immediately after encapsulation was observed. For some applications, and in particular to determine the amount of released A $\beta$  over time, it is important to predict the number of cells encapsulated in each alginate bead.

Interestingly, the encapsulation process does not have a significant impact on cell viability. Results are reported in a previous work, in which colorectal cancer cells (i.e., HCT-116) were encapsulated using a similar method, with no differences in cell viability in alginate microbeads compared to 2D controls<sup>20</sup>.

To measure the stability of the alginate used in the encapsulation process, we measured microbead diameters over a 14-day period (n = 100). There were no observed changes in average diameter 14 days after encapsulation as compared with immediately after encapsulation (data not reported).

#### **Encapsulated 7PA2 cells release A $\beta$ over time**

Conditioned media analyzed from 2D and 3D cultures of 7PA2 cells reveal a constant increase in A $\beta$ <sub>1-42</sub> levels. Cell culture media was sampled every 24 h, and up to four days, and analyzed using ELISA. Our data shows that the rate of release of A $\beta$ <sub>1-42</sub> from microbeads (3D) is similar in profile to that released from the 2D culture (**Figure 4**).

[Insert Figure 4]

#### **Potential application of encapsulated 7PA2 cells**

We report that 7PA2 cells encapsulated in alginate microbeads can be effectively used for the sustained release of A $\beta$ <sub>1-42</sub>, and hence used to test the effect of chronic A $\beta$  secretion in any preclinical model. In **Figure 5** below, we show the advantages of using alginate microbeads that can be easily injected and located within the brain of a rat. Ex vivo sections are here used for illustration purposes, comparing a millimeter alginate bead versus fabricated alginate microbeads.

[Insert Figure 5]

The images above highlight the importance of controlling the size of the microbead for such studies. Here, we demonstrate the advantage of using microbeads of diameters under 600  $\mu$ m. This allows the use of minimally invasive injection (e.g., Hamilton syringe) to better control the location of injected beads within the brain.

In summary, encapsulating 7PA2 cells gives control over the size of microbeads, number of encapsulated cells and the prediction of A $\beta$  secreted from the microbeads (e.g., concentration, release profile). Controlling the size of the microbeads is essential for two reasons: 1) to permit fine-tuned control over the concentration of released A $\beta$ , and 2) to allow implantation in a controlled region of the rat brain. The results obtained here describe the facile tuning of alginate microbeads and highlight potential applications for further studies.

#### **FIGURE AND TABLE LEGENDS:**

**Figure 1: The encapsulation system.** (A) Schematic representation of the encapsulation system. An alginate-cell suspension is loaded into a syringe (2) and fed through a reservoir at an extrusion speed set at syringe pump (1). In the reservoir, a vibration hat (3) vibrates at a frequency set by a waveform generator (4) to disrupt the stream at equal intervals, forming equally-sized droplets. As the solution is fed through a nozzle (5) and droplets are formed, an electrostatic potential is applied across an electrode (7) set by a voltage generator (6), which slightly charges the surface of the droplets, allowing the stream to spread as a result of repelling electrostatic forces. As

droplets engage with the gelation bath (8),  $\text{Ca}^{2+}$ -driven cross-linking of alginate results in the formation of spherical microbeads. (B) Photograph of the encapsulator before fabrication of alginate microbeads.

**Figure 2: Fabrication method optimization.** (A) Photographs showing changes in stream dispersion. (B) Bright-field image showing alginate spherical microbeads immediately after fabrication. (C) Example of optimization step: tuning selected fabrication parameters to achieve the target microbead size. Selected graphs to illustrate the relationship between each parameter and the size of the resultant microbeads (size distribution from at least  $n = 100$  microbeads). Note that the nozzle internal diameter, viscosity of ejected solution and gelling conditions can also influence the size of fabricated beads. Error bars represent S.D.

**Figure 3: Encapsulated 7PA2 cells.** (A) Bright-field image of encapsulated 7PA2 cells showing even cell distribution throughout the alginate microbead. Fabrication parameters were set to obtain  $\sim 150$  7PA2 cells per bead. No significant variation in alginate beads was observed over 7 days of culture. (B) There was no observed difference between the overall proliferation of 7PA2 cells incubated with alginate versus cells incubated without alginate. 7PA2 cells grow and migrate across the volume of the alginate beads; reduction of serum concentration can be considered to slow down cell growth. Error bars represent S.D.

**Figure 4: Rate of  $\text{A}\beta_{1-42}$  secretion from 7PA2 cells.** (A)  $\text{A}\beta$  release (% normalized after 4 days) from 2D and 3D in vitro models has a similar profile. Both models show a constant increase in  $\text{A}\beta$  levels over the four-day period and, as expected, a steady concentration is not reached. (B) Concentration of  $\text{A}\beta_{1-42}$  normalized to initial cell number, showing similar secretion of  $\text{A}\beta_{1-42}$  over time, regardless of the culturing methods. Neither the presence of alginate nor the process of encapsulation (3D in vitro model) alter the secretion of  $\text{A}\beta_{1-42}$ . Error bars represent S.D.

**Figure 5: Using microbeads for relevant preclinical applications.** Microbeads for engraftment in the rat brain must be small enough to be embedded without creating a lesion too large to avoid damage to the brain parenchyma and adversely affect normal brain function. Image (A) shows a size comparison between a millimeter-scaled bead and a micrometer-scaled bead side by side. (B) Implanting a millimeter-scaled bead within the brain for in vivo purposes would not work. Image (C) shows a microbead fabricated using this protocol. The size is suitable for insertion within the hippocampus of the rat without having a detrimental effect on normal physiology.

**Figure 6: Predicted release profile of  $\text{A}\beta_{1-42}$  from encapsulated 7PA2 cells compared with a bolus injection.** The profile of  $\text{A}\beta$  release from engrafted 7PA2-containing microbeads allows the testing of the effects of chronic and sustained  $\text{A}\beta$  in an animal model of relevance to AD. Conversely, a bolus injection would create a spike in  $\text{A}\beta$  levels over a short period of time, followed by a rapid clearance of  $\text{A}\beta$  from the brain.

**Table 1: Fabrication parameters and their influence on microbead size.** The table illustrates how each parameter can influence the resultant size of fabricated microbeads, irrespective of the nozzle and the viscosity of the solution used.

## DISCUSSION:

The method outlined in this article is useful for encapsulating cells achieving a narrow size distribution of alginate microbeads<sup>27</sup>. It also affords the advantage of growing cells in an immunologically isolated environment<sup>17,19</sup>, protecting them from external stress. Additionally, encapsulation of cells in alginate more closely mimics physiological conditions, specifically with regards to cell-to-cell interactions and matrix stiffness<sup>20</sup>. These factors are all particularly crucial for subsequent use in relevant applications, such as in vivo engraftment, precluding potential immune reactions in the surrounding tissue<sup>17</sup>. Furthermore, a major advantage of this protocol is the easy tuneability according to the application of interest: it is possible to modify the protocol and optimize the parameters for fabrication of larger or smaller, and softer or stiffer beads. The described method is used to fabricate beads small enough to be injected with minimally invasive methods, and sufficiently large enough to host a number of cells and provide a sufficient release of A $\beta$  to result in observable behavioral and pathological effects upon injection in animal models.

The success of this protocol is dependent on a number of critical steps. Careful cell handling and optimal cell culturing techniques are important to maintain cell viability and function<sup>20,28</sup>. Using standard culture conditions ensures the conservation of normal function of 7PA2 cells, as observed. This allows a similar release profile for A $\beta$ <sub>1-42</sub> from encapsulated cells when compared with 2D cultures. In addition, for the protocol to work optimally, a low-viscosity alginate solution guarantees better results compared with a high viscosity alginate solution. This ensures that a homogenous laminar jet is extruded through the nozzle and an even distribution of cells within the matrix of the fabricated beads<sup>27</sup>. Materials used to encapsulate cells must have a very rapid gelation mechanism, allowing retention of shape.

Another critical step in this protocol is the handling of fabricated microbeads following gelation. Here, we show the retrieval of microbeads by using a large aperture plastic pipette to transfer the beads. Alternatively, pouring the calcium chloride solution containing the microbeads into a mesh filter can be used for bead retrieval. A large (5, 10 or 25 mL) serological pipette can be used to draw up the microbeads and then washed through the mesh filter instead of pouring. The advantage of this is a higher confidence in the sterility of the procedure compared to pouring. However, one of the limitations is that some beads may be distorted if they are compressed by the pipette, in addition to risking a lower yield if a large proportion of microbeads are not rescued.

This approach has been used to encapsulate different cell lines to model and study different diseases (e.g., release of insulin from encapsulated pancreatic islet). The novelty of our approach is the engraftment of the microbeads generated using this protocol as a useful method in modeling important aspects of Alzheimer's disease in vivo. When comparing the release profile of A $\beta$  from encapsulated cells (**Figure 4**) to the levels of A $\beta$  generated by a bolus injection (such as that reported in other studies<sup>3,26</sup>), a more chronic and sustained release of A $\beta$  can be expected. **Figure 6** illustrates the predicted tendency that may be achieved. Using this system for in vivo modeling is more relevant to the way the disease progresses and can be more useful in drug discovery and development.

[Insert Figure 6 here]

## ACKNOWLEDGMENTS:

The authors would like to thank Mr Kajen Suresparan, Dr Jonathan Wubetu, Mr Dominik Grudzinski, Miss Chen Zhao, and Dr Thierry Pilot for their help in the optimization of alginate microbead fabrication, cell culture and A $\beta$  detection, and useful scientific discussions.

## DISCLOSURES:

The authors have nothing to disclose.

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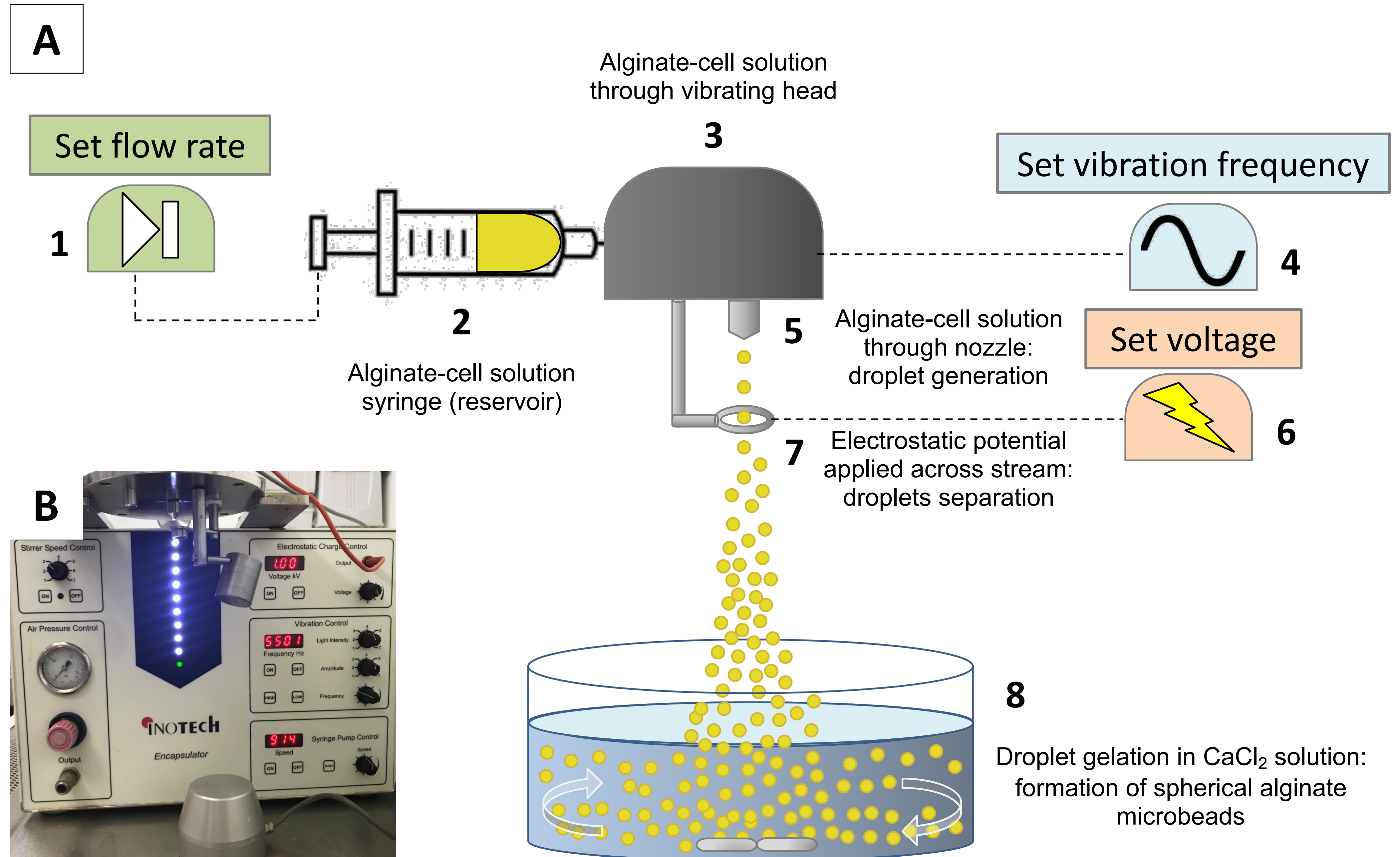
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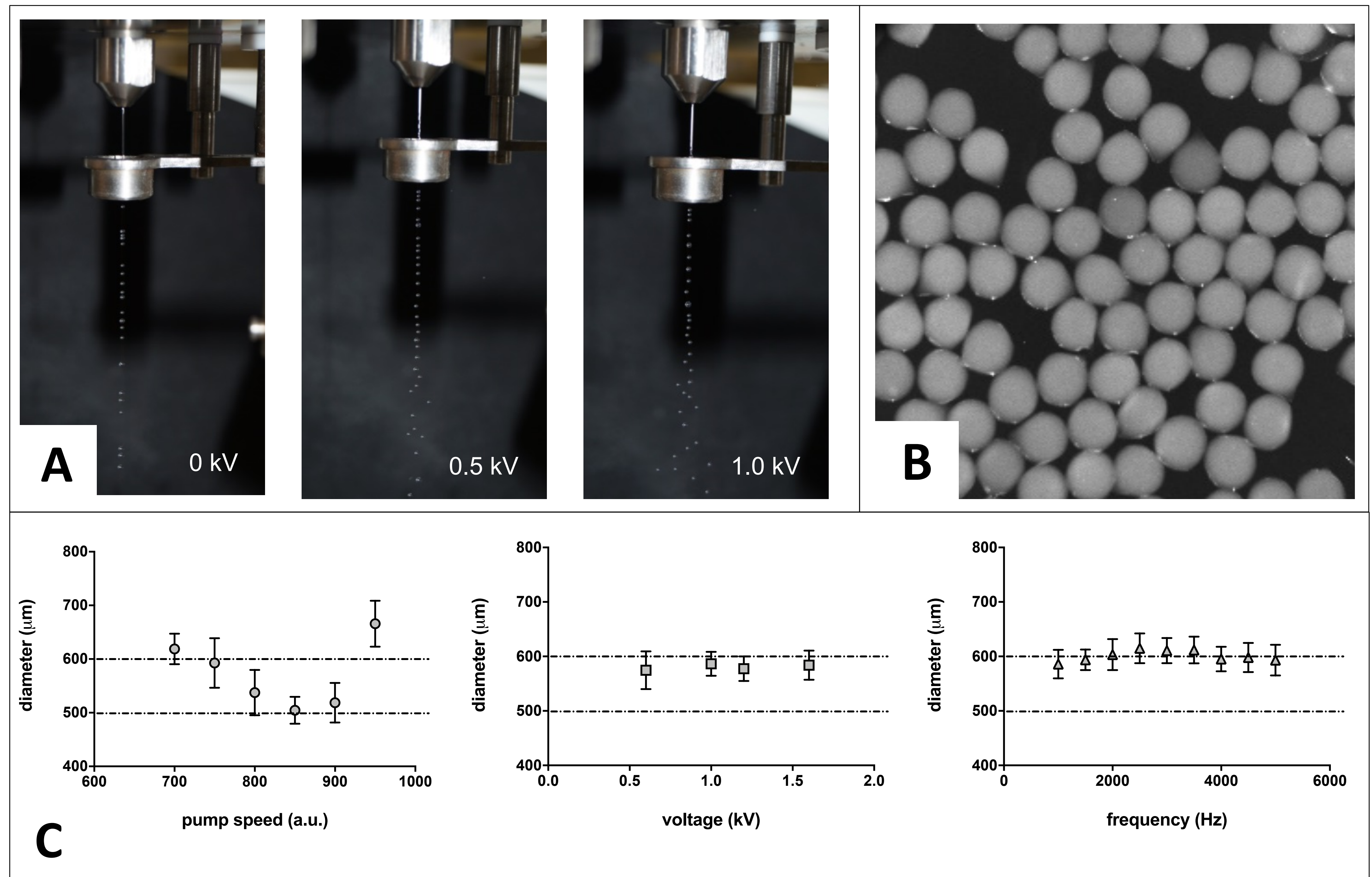
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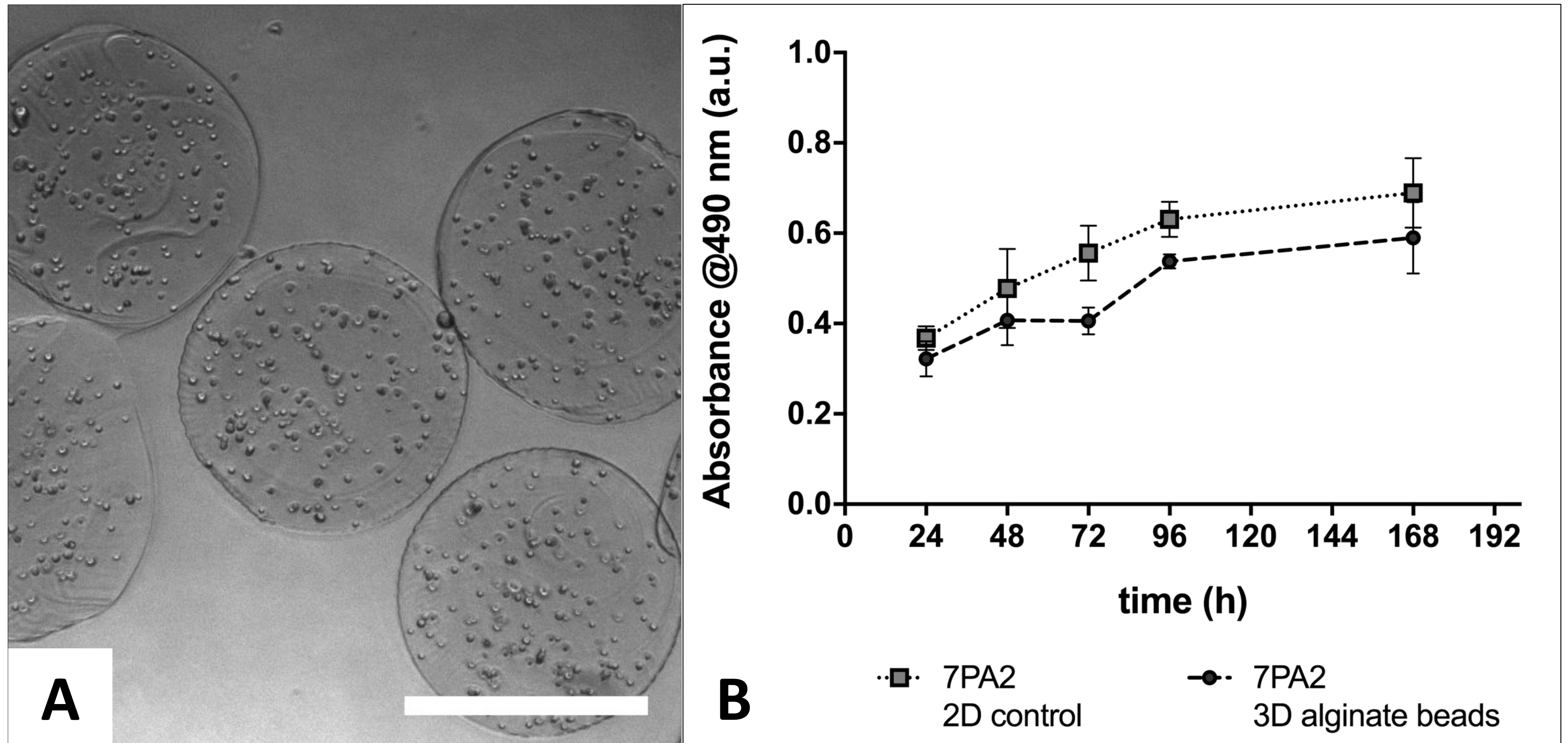


**Figure 1**

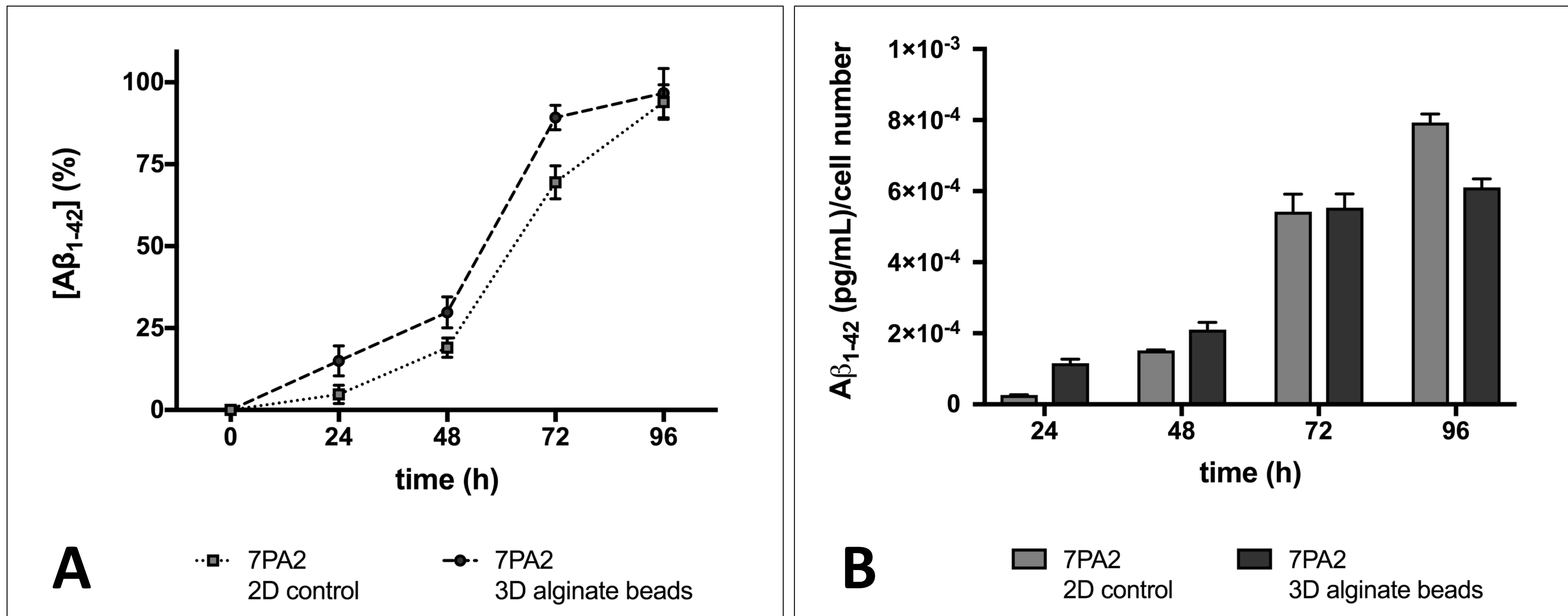


**Figure 2**



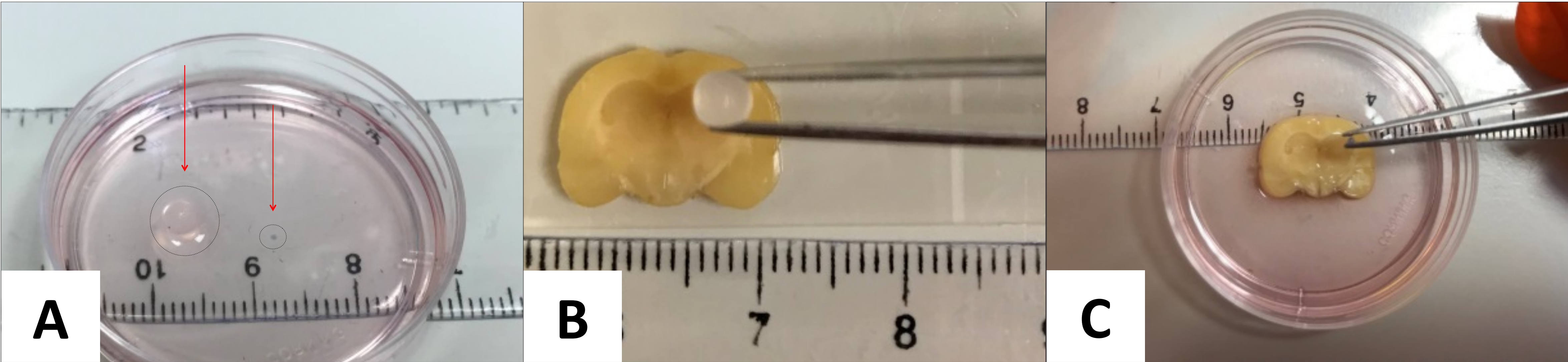


**Figure 3**

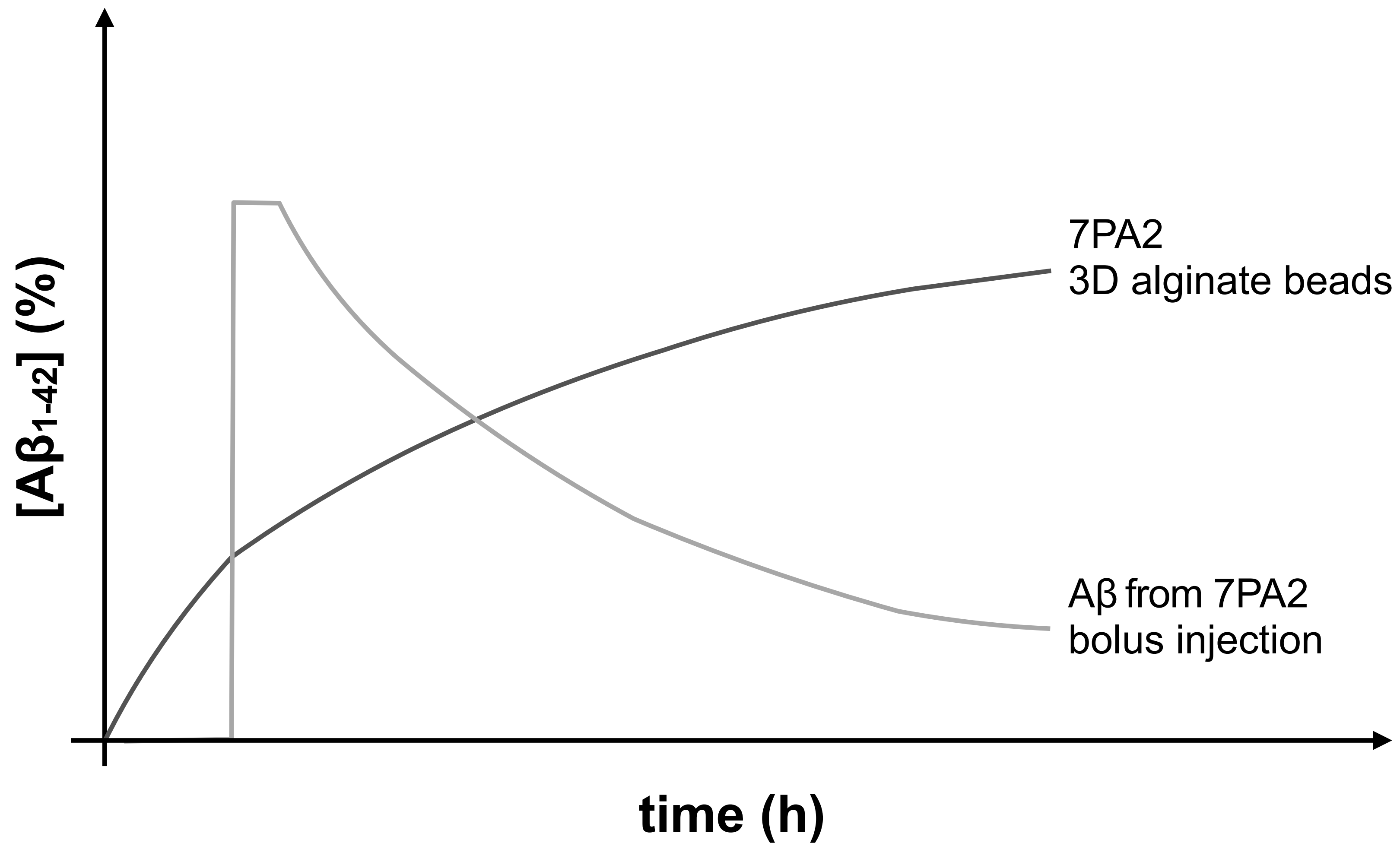


**Figure 4**





**Figure 5**



**Figure 6**

Table 1

Parameter	Nozzle size	Vibration frequency	Flow rate	Electrode voltage
Bead size	<div>↑ ↑↑</div>	<div>↑ ↓</div>	<div>↑ ↑</div>	<div>↑ –</div>

Name of Material/Equipment	Company	Catalog Number	Comments/Description
µManager software	Vale Lab, UCSF, USA	v.1.46	
0.22 um PES filter	Merck, UK	SLGP033RS	
15mm Netwell insert 74 um mesh filter	Constar, usa	#3477	
Alginate acid sodium salt from brown algae	Sigma-Aldrich,uk	A0682	
Calcium chloride	Sigma-Aldrich,uk	C1016	
CellTiter96 AQueous One Solution cell proliferation assay	Promega, USA	G3580	
Encapsulator	Inotech	IE-50	serial no. 05.002.01-200
HEPES	Sigma-Aldrich,uk	H4024	
Hu Aβ 1-42 ELISA	ThermoFisher, UK	KHB3441	
ImageJ software	ImageJ	v1.49p	
Inverted light microscope	Olympus	CKX41	
Leica microscope	Leica microsystems, UK	DMI6000B	
Neo sCMOS Camera	Ander, UK		5.5
Phosphate buffered saline	Sigma-Aldrich,uk	D1408	
Sodium chloride	Sigma-Aldrich,uk	433209	
Trisodium citrate dihydrate	Sigma-Aldrich,uk	W302600-K	
Trypsin-EDTA solution	Sigma-Aldrich,uk	T4049	





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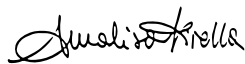
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(I comment by typing the original lines and then the suggestion for change)

Very, very nice piece of work.

In conclusion, our optimised method for the production of stable alginate microbeads containing amyloid-producing 7PA2 cells enables the modelling of important aspects of AD both in vitro and in vivo.

In conclusion, our optimised method for the production of stable alginate microbeads containing amyloid-producing 7PA2 cells might enable the modelling of important aspects of

AD both in vitro and in vivo.

Explanation: You have not tested in vivo so you do not know if it works (I do believe it will)

**Amended. Thanks for noting this.**

74 A $\beta$  peptides in high concentrations deposited all in one go. This is inconsistent with the chronic,

75 sustainable nature of the release of A $\beta$  in disease5.

74 A $\beta$  peptides in high concentrations deposited all in one go. This is inconsistent with the chronic,

75 sustained nature of the release of A $\beta$  in disease5.

**We agree with the reviewer. Thanks for noting this.**

91 earlier changes exhibited in response to the chronic and sustainable exposure to the A $\beta$

91 earlier changes exhibited in response to the chronic and sustained exposure to the A $\beta$

**Amended.**

97 To this end, we have developed a system which allows the constant, sustainable secretion of A $\beta$  IDEM!

**Idem as above.**

103 Alginate was the selected biomaterial as it is biocompatible, non-degradable and does not

Non degradable? May be in your scenario but it is degradable, at least in the human large intestines.

**We agree with the reviewer. Thanks for noting this. We amended the manuscript accordingly.**

124 Val717Phe mutated gene23. These cells continually produce catalytic products of APP, including

I am in doubt, is it Val717Phe or v717f? Why not use (or mention) both notations to make sure the article is found anyway?

**Both notations are acceptable. We are happy to change to V717F.**

1. Prepare 100 mL alginate 4% (w/v) stock solution

182 1.1. Weigh 4.4 g alginate sodium salt, and add the dry powder in 100 mL of HEPES buffered

183 saline (HBS, 20 mM HEPES and 150 mM NaCl in MilliQ water) allowing dissolution.

184 1.2. Use a magnetic stirrer at ~500 rpm and heat to up to 50 °C to allow complete alginate

185 dissolution. Note: the alginate may need one or two hours to dissolve completely. To save

186 on time, alginate solution can be made up a day in advance of the encapsulation protocol

187 and stored at 4 °C until the next day. When alginate has been refrigerated, heat gently to

188 37 °C using a water bath or a hot plate and agitate with a magnetic stirrer immediately  
189 before use.

A few comments: you cannot write that it is 4% w/v unless you have actually measured the FINAL volume to be 104.4 (which I doubt you did). I suggest you say "approximately 4%" (it is not very important because you describe the precise procedure but, it is scientific paper...)

We agree with the reviewer. Thanks for noting this. We amended the manuscript accordingly.

Then, I object to the monikers "solution", "dissolve completely" and "complete alginate Dissolution". What you have is a SOL, the polymer is HYDRATED, hopefully to COMPLETE HYDRATION. When Calcium is added you achieve a SOL-GEL TRANSITION.

I know, many use the wrong terms but it would be refreshing to see the correct nomenclature.

In line with all the recent literature, we would like to keep the used terms. We agree to change 'dissolution' to 'hydrated'. 'Physical gelation' is commonly accepted, and easier to understand than 'sol-gel transition' to a broader audience.

208 4.3. Attach 300 µm nozzle and repeat 4.2.

208 4.3. Attach a (or the) 300 µm nozzle and repeat 4.2.

Amended.

209 4.4. Spray the bottle containing the filtered alginate solution, bottle containing filtered calcium

209 4.4. Spray the bottle containing the filtered alginate sol, the bottle containing filtered calcium

Amended.

243 7.5. Upon contact with the gelation bath, the alginate droplets will instantly cross-link with the

244 calcium ions in the gelation bath and form spherical microbeads.

243 7.5. Upon contact with the gelation bath, the alginate in the droplets will instantly cross-link 244 with the calcium ions in the gelation bath and form spherical microbeads.

(the polymer cross-links, not the droplets)

Amended.

261 cells. Note: this only needs to be performed on a sample from each microbead population.  
261 cells. Note: this only needs to be performed on a single sample from each microbead population.

Amended.

269 imaging software. Dramatic subsequent variations in diameter can be indicative of alginate

270 degradation.

269 imaging software. Dramatic subsequent variations in diameter can be indicative of alginate

270 degradation. (You see? You wrote that alginate is non-degradable :-))

Above statement has been amended.

435 compared with 2D cultures. In addition, for the protocol to work optimally, a low-viscosity

436 alginate solution guarantees better results compared with a high viscosity alginate solution.

We miss the reviewer's point here.

AND FINALLY:

409 In conclusion, encapsulating 7PA2 cells gives control over the size of microbeads, number of

410 encapsulated cells and the prediction of A $\beta$  secreted from the microbeads (e.g. concentration,

411 release profile). Controlling the size of the microbeads is essential for two reasons: 1) to fine

412 control the concentration of released A $\beta$ , and 2) to allow implantation in a controlled region of

413 the rat brain. The results obtained here describe the facile tuning of alginate microbeads and

414 highlight potential applications for further studies.

This paragraph is the conclusion. The dictionary says:

Conclusion:

the end or finish of an event, process, or text.

"the conclusion of World War Two"

synonyms: end, ending, finish, close, closure, termination, wind-up, cessation; More

So, it should be AFTER the discussion!

The word 'conclusion' has been changed to 'summary'.

**Reviewer #2:**

Line 174 : You have to review the principle of the process. The size is mainly function of nozzle size. All other parameters must have very limited impact as you have to select optimum value which is linked to the nozzle size.

We agree with the reviewer that the nozzle diameter has a big impact on beads size. However, the other parameters are also important to achieve a good size distribution (low polydispersity index) and shape. We have clarified the statement to reflect this.

Line 182 : you have to specify which alginate you used, at minimum the viscosity and guluronic content

These characteristics are now included in the text. Nevertheless, they are available online in the manufacturer's datasheet.

Line 184 : No interest to warm up alginate. It dissolves at room temperature

Our point here is to speed-up the hydration process. Warming up the alginate solution is also important to better control the homogeneity of cell suspension and reduce the viscosity of the suspension for further extrusion and beads formation.

Line 230 : Looking at Figure 2, you are probably not at fully optimum conditions (droplets are not uniformly distributed along the droplet stream)

We believe that we achieved optimum conditions. Images are used here to illustrate the formation of the droplets with this system.

Line 246 : 4 minutes is quite limited. Generally people let stand for 15 minutes

We believe that exposing cells to calcium for longer than 4 or 5 min would impact on their behaviour due to calcium exposure. Stability of alginate gels prepared with this procedure and the resultant mechanical properties are not affected (results not reported). We believe that this gelling time is sufficient to form stable gels for the purpose of the proposed study. Optimisation in this respect can always be performed for different applications.

Line 308 : see comment on line 174.

See our reply to that comment.

Line 318 : generally is expected that the internal diameter of the needle must be 3 times the bead diameter but if it work for you ...

Thanks for the comment. From our experience we have not noticed any significant bead disruption while extruding through a 20G needle.

Line 359 : The difference between theoretical and experimental values are in the range of error. Then you may expect that they are not really different. But did you take in account the contraction of the beads during gelation. I get myself 131 based on concentration in alginate solution but the contraction may be up to 50 % leading to 262 cells per beads

This is a good point. By using 4-5 min to allow physical gelation of cell-alginate suspension, we do not observe a large shrinkage of the hydrogel.

Here we used this theoretical estimation of cell number to check the quality of the encapsulation process. This could be useful in future study, in which cell concentration is trivial to control A $\beta$  release. We thank the reviewer for the comment, and we will consider this aspect in the future, if we will change the gelation conditions.

Line 378 : Unit of release rate ? For me the value is a little bit higher in 3D system ... which quite positive

This is a normalised concentration of A $\beta$  per cell no.

We agree with the reviewer that a 3D environment has an impact on the release of proteins. However, this could be a positive, negative or neutral effect; in our study we did



not observe an overall significant difference between 2D and 3D in the time scale reported (4 days).

Figure 2 A : The effect of the voltage is to disperse the beads to avoid collision and coalescence. It do not modify the flow.

We agree in principle, and we do in fact report that the size does not change significantly once the voltage is varied. However, we do think that voltage affects the final microbead size and size distribution. We also believe that the homogeneously dispersed flow plays a significant role in preserving the shape of formed beads.

Figure 2C : start your Y-axis at zero to avoid insignificant apparent effect. Voltage and frequency has no effect. But theoretically, size must be proportional to  $(\text{flow rate})^{1/3}$  and  $(1/\text{frequency})^{1/3}$ . However, if you do not work at optimum conditions (nozzle size --> flow rate/frequency) you will not get monodisperse beads

We miss this point. What is your comment about?

Figure 6 : Is it just a tendency graph or an experimental one ?

This graph is included to show the trend of A $\beta$  concentration between two different administration approaches. It is a tendency graph, we clarified this both in the manuscript and in the figure caption.

Reviewer #3:

Manuscript Summary:

In this manuscript the authors describe a method for the encapsulation of amyloid- $\beta$  secreting 7PA2 cells in alginate microbeads. The encapsulation of these A $\beta$  secreting cells has many potential benefits in both in vivo and ex vivo Alzheimer's disease research which are addressed in the introduction. Using a cell encapsulation device the authors demonstrate a simple method for the uniform encapsulation of the 7PA2 cells in alginate microbeads ~500-600  $\mu\text{m}$  in size. The authors then present representative results that confirm that these encapsulating cells enable the continuous production of A $\beta$  and are a form factor more amicable to implantation into the rat brain. Overall this is a well written manuscript that describes a cell encapsulation method that could be of interest to a wide audience. However, I do believe that more detail should be given in the protocol before accepting.

Major Concerns:

A major concern with manuscript is that the protocol is too specific. There are two ways in which this protocol could be broadened to attract a wider audience.

- 1) As it is now this protocol covers only the encapsulation of the cells in a specific microbead size, and not a wide range of sizes. This protocol would be much more interesting to a wide audience if there was a table of values that can be used with this protocol to create a wide range of microbead sizes. Although this is presented for some parameters in Figure 2, it would be better to have this presented in the protocol itself as a table of values to be used for different size ranges.

2) If the authors want to focus the protocol more on this specific size of microbead and its use in Alzheimer's disease models then animal methods (implantation/analysis etc) should be added to the protocol in addition to the fabrication process that is there now.

We agree with this comment. In fact, there are several applications of hydrogel beads encapsulating different cell types. For this specific encapsulating system, we report two different cell types, one reported here and another already published and referenced in the manuscript. We have now included in the first part of the manuscript how different parameters can be varied to achieve different sized microbeads. (Page 5, line 182)

	Nozzle size ↑	Vibration frequency ↑	Flow rate ↑	Electrode voltage ↑
Bead size	↑↑	↓	↑	–

#### Minor Concerns:

Page 5 - Line 198 - 199: As you have given the gram amounts for the other molar solutions made in this protocol thus far it would be good to include those here.

We have included these values in the revised manuscript.

Page 6 - Line 231 - 232: As mentioned in the major concerns a table in this section illustrating different parameters to obtain different microbead sizes would be very beneficial for this protocol to appeal to a wider audience.

See reply to the major concerns comment. (Page 5, line 182)

Page 7 - Lines 254 and 261: "Note:" should be consistently bolded in the manuscript.

Amended. Thanks for reporting.

Page 9 - Line 388: "Note:" should be bolded in figure caption.

Amended. Thanks for reporting.

Page 9 - Line 342 - 343: The part of the sentence "...microscope analysis was performed on microscope images" sounds redundant as it is written now.

Amended. Thanks for reporting.

It appears that the manuscript is currently written in British English. This should be changed to American English as per JoVE standard.

Amended. Thanks for reporting.

### **Reply to Editorial Comments:**

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

*Thanks. We reviewed the manuscript and correct spelling or grammatical error. Major changes are tracked with red font.*

- **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

*We checked the protocol and we believe that now all the necessary details are now included in the manuscript.*

- **Protocol Numbering:** There must be a one-line space between each protocol step.

*One-line space is now included between each protocol step.*

- **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

*The updated version of the manuscript now contains highlighted parts/steps to be visualised. We believed these steps are the critical ones to display the experimental procedure described in the manuscript.*

- **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

*We followed JoVE's guidelines and changed the Discussion section accordingly. Changes are tracked with red font.*

- **References:**

- 1) Please spell out journal names.
- 2) Please fix references 11, 22, 28 and ensure they match the style format below.
- 3) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

*We changed the references as suggested.*

- **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MilliQ, Virkon,

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*We changed all the commercial names specified (i.e. MilliQ, Virkon)*

- Please define all abbreviations at first use.

*Modification implemented.*

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