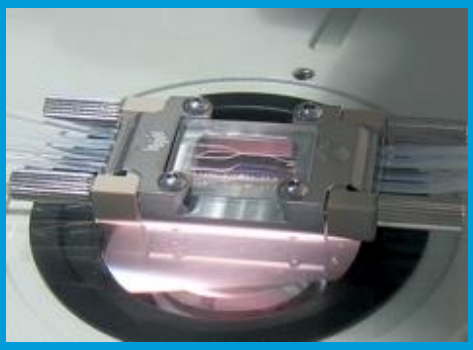
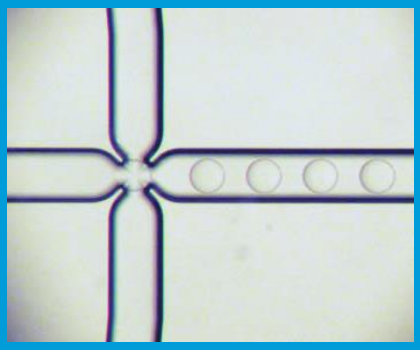
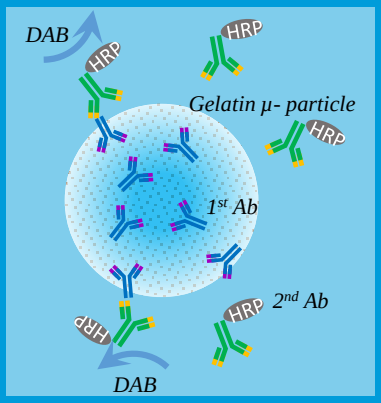
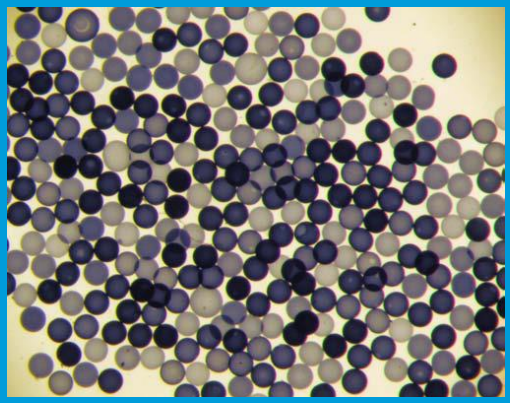
Colorimetric Estimation of Protein Content in Gelatin Microparticles

Production & Subsequent Quantification of Protein Seeded Gelatin Microparticles Using Advanced Droplet System



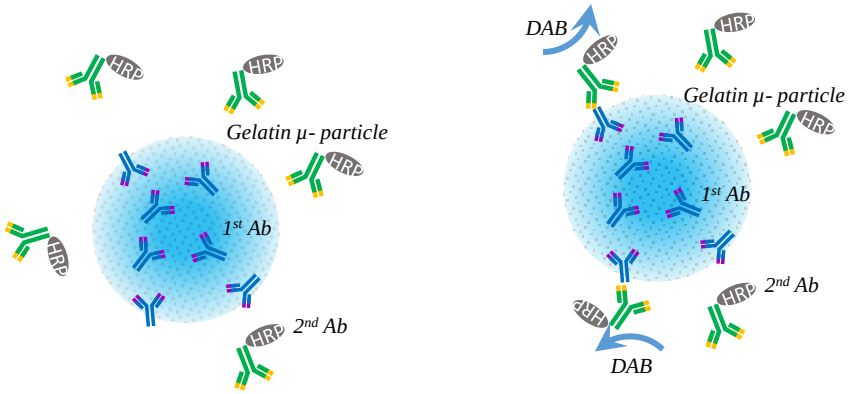






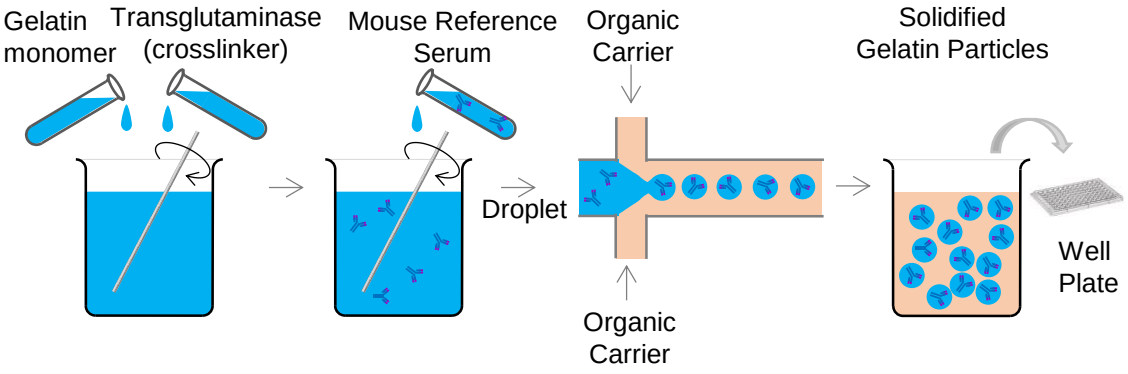
Summary

This application note demonstrates a colorimetric method to quantify protein content inside micrometer sized gelatin hydrogel particles. Here, an HRP-coupled antibody (2nd *Ab*) is used as a probe which activates a visual signal on coming in contact with a Serum antibody (1st *Ab*) – a blue color is emitted. The 1st *Ab* is embedded inside the hydrogel matrix of the microparticle. The objective is to quantify surface exposed antibodies (1st *Ab*) via a color reaction using *3,3'-Diaminobenzidine* (DAB).



Protein quantification – colorimetric detection by HRP-DAB coupled secondary antibody binding with reference serum (containing IgG mouse antibodies).

Two different concentrations of 1st *Ab* are seeded in Gelatin microparticles, which then are expected to generate different intensities of blue color. The intensity of the blue color is indicative of the quantity of protein at the surface of the particle, which in turn is dependent on the quantity contained within the core of the particle.



Schematic for production of gelatin particles containing protein (mouse serum antibodies – 1st Ab). A microfluidic flow focussing method produces highly monodisperse particles. The final product is transferred to a well-plate.

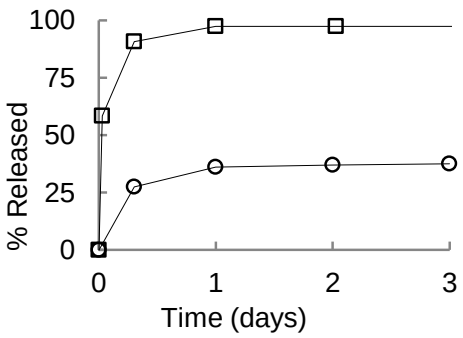
If a reaction is positive and if signal differences are detectable, then the method can be used to evaluate protein content in droplets. The protein itself may either originate from direct encapsulation in droplets, or be an outcome of cell activity from encapsulated cells. The tests were done at room temperature as well as at lowered temperature (-20°C) to assess any differences brought about by temperature.

Therapeutic Protein Administration

Novel aspects for administration of therapeutic proteins and peptides are needed. The gastro-intestinal tract for instance has various barriers for the delivery, such as proteolytic degradation – resulting in degradation of the compound prior to absorption and the inability of the macromolecules to penetrate the intestinal cell wall. In other cases, microencapsulated genetically engineered whole cells release therapeutic proteins as part of the delivery application. In either case, the functional requirement of the carrier of is to protect the protein structure that is essential for preserving the bioactivity of the protein.

Gelatin Material as Protein Transport Medium

With controlled release and sustained delivery rates, maintaining concentration within therapeutic limits is possible. At the same time, toxicity related systemic side effects can be minimized. The use of hydrogel microparticles is useful in these situations of continuous and controlled drug administration.



In vitro release profiles of a common protein from hydrogels. The rate of release depends strongly on the mechanical properties of the solidified particles.

Gelatin being a denatured, biodegradable protein is one of the popular selections for being a protein carrier. When mixed with positively or negatively charged gelatin, an oppositely charged protein will ionically interact to form a polyion complex*.

When considering the diffusion of ions and molecules in solutions, it is generally useful to be able to estimate the time required for diffusion over a given distance. From a physiological perspective, this knowledge helps us better understand how long it takes molecules and ions to travel physiologically relevant distances by diffusion alone.

$$t = \frac{x^2}{2D}$$

- ***D*** (= 4.0 ×10^{-7}) is the diffusion coefficient of a solute in free solution
- ***x*** (= 100 µm) is the mean distance travelled by the diffusing solute in one direction along one axis after elapsed time ***t***.
- ***t*** (= 2.083 s) is the elapsed time since diffusion began, considering no controlled rate method.

A diffusion coefficient has been determined experimentally for the following system. HEK293 cells genetically engineered to secrete interleukin-2 (hIL2) 4% mTG-gels Gelatin

* *Y. Tabata, Y. Ikada.* ***Protein release from gelatin matrices****. Advanced Drug Delivery Reviews, (1998), 31, 287-301.*

hydrogel cross-linked using microbial transglutaminase (mTG) The diffusion coefficient[†] of hIL2 through the hydrogel D_m = 4.0 x 10-7 cm^2/s. The molecular weight of hIL2 is similar to that of IgG.

Ion/Molecule	Atomic/Molecular Weight (g/mol)	Diffusion Coefficient (cm^2/s)
Na^+	22.990	1.33×10^{-5}
K^+	39.098	1.96×10^{-5}
Ca^{2+}	40.078	0.79×10^{-5}
Oxygen (O_2)	31.999	2.10×10^{-5}
Carbon dioxide (CO_2)	44.01	1.97×10^{-5}
Urea	60.055	1.38×10^{-5}
Glucose	180.156	5×10^{-6}
Sucrose	342.296	5.23×10^{-6}
Haemoglobin	68,000	6.9×10^{-7}
DNA	≈ 6,000,000	1.3×10^{-8}

Diffusion coefficient values for selected ions and small and large molecules. The diffusion coefficient varies with temperature and is also a function of the medium in which diffusion occurs. The values shown are for diffusion in water (H_2O) at 25 °C.

HRP Test – Quantification of Protein Content Inside Gelatin Hydrogel

Western blot and ELISA are established analytical techniques for the specific detection of proteins in samples (cells, tissues). In either technique, protein-specific antibodies (primary antibodies – 1st *Ab*) interact with a specific target (a conjugated secondary antibody – 2nd *Ab*). HRP detection is an economical method using *3,3'-Diaminobenzidine* (DAB)[‡]. The protein is detected using a secondary antibody that is conjugated to *horseradish peroxidase* (HRP). When the enzyme substrate is added, a colored precipitate is deposited on the substrate. As the precipitate accumulates, a colored signal develops and is readily visible by eye on the surface of the microparticle. The enzymatic reaction can be monitored and stopped when the desired signal over background is reached.

Why Monodisperse?

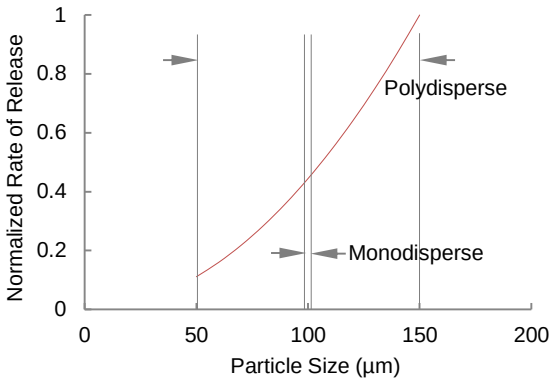
There are various microsphere preparation methods, however, the conventional bulk methods often result in polydisperse microspheres with poor encapsulation efficiencies.

[†] *Chong Wing Yung, William E. Bentley, Timothy A. Barbari (2010).* ***Diffusion of interleukin-2 from cells overlaid with cytocompatible enzyme-crosslinked gelatin hydrogels****. J. Biomed. Mat. Res. A, (95A), 25-32.*

[‡] *Tsang VC et al. (1985).* ***Enzyme-linked immunoelectrotransfer blot (EITB). In Enzyme-Mediated Immunoassay.*** *T.T. Ngo and H.M. Lenhoff, eds. (New York: Plenum Press), pp 389–414.*

The resulting product contains particles of large size distributions. The resulting release rates are then unpredictable as the rate of release depends on the exposed surface area. The exposed surface area varies as the squared of the droplet diameter.

The schematic to the right depicts the variability in the release rate (on a normalized scale) as a function of droplet sizes. For a polydisperse droplet sample, the droplet sizes vary between 50 µm and 150 µm diameter. On a normalized scale, this indicates a release rate variability of between 0.2 and 1.0, which is quite large. A monodisperse sample on the other hand has droplet with sizes varying between (say) 95 µm and 105 µm. The corresponding range of release rates is between 0.4 and 0.5.

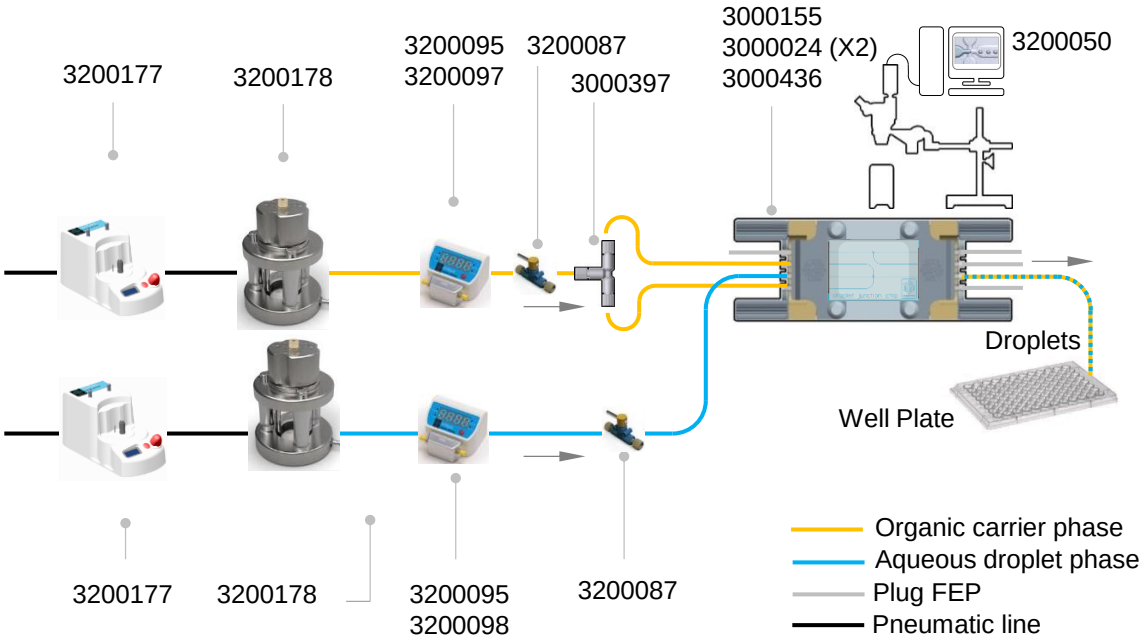


Rate of release can vary by as much as 10X due to polydispersity of particles. Monodisperse particles have a narrower range of release rate.

Optimization of the microcapsules with regard to mechanical stability, cell growth, and secretion of proteins is necessary in order to evaluate the future use of this delivery technology. In another application note, we have explored the encapsulation and viability of CHO cells encapsulated in droplets over a period of three weeks with good results. This application note addresses quantification of proteins that are imbibed into the gelatin hydrogel.

Droplet System Setup

Dolomite's Droplet System is used to manufacture the microstructured gelatin carriers used in this study. The pressure based pumping enabled by the P-Pump is critical to allow generation of highly monodisperse droplet sizes. A schematic is presented below showing the major components of such a system.



Schematic showing parts used for test setup. The P-Pump and sensors on each line are coupled to enable flow-control mode of pumping. PC not included.

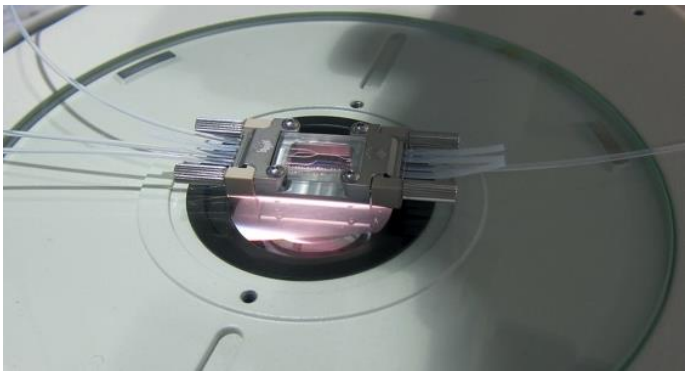
The pumps were controlled using Flow Control Center – the software interface – and run in pressure control mode. The microfluidic device utilizes a flow focussing junction geometry where the droplet phase is non-wetting while the carrier phase wets the channel surfaces. A high magnification microscope continuously monitors the device junction. With consideration to balancing the fluid pressures at the droplet junction, the tubing sizes are selected to provide the optimal fluidic resistance. These are specified in the accompanying table. All tubing is made of FEP, and outer diameter of all tubing is 1.6mm.

	Organic Carrier ID(mm); L(mm)	Aqueous Droplet ID(mm); L(mm)
Pump to Remote Chamber	Pneumatic tubing	Pneumatic tubing
Remote Chamber to Sensor	0.25; 300	0.10; 500
Senor to T-connector	0.25; 300	0.10; 1000
T to chip	0.25; 500 mm (×2)	
Chip to Outlet	0.25; 500	

The OD of all tubing used is 1.6 mm. Fluidic resistances were calculated according to the Microfluidic Wizard on the Dolomite Website[§].

[§] **Microfluidic Calculator** *http://www.dolomite-microfluidics.com/en/applications/application-notes/172*

The Pico-Surf™ 2 based on fluorocarbon oil has high gas solubility. It has been seen that when Nitrogen or air is used to pressurize the P-Pumps, the gas dissolves into the Pico-Surf™ 2 at high pressure. Downstream, when the pressure falls, the gas degasses out and appears as bubbles which grow progressively larger as they travel downstream. Often these bubbles are harmless, but other times may cause fluidic disturbances. To counter this, the use of Helium greatly reduces and often eliminates the degassing problem.



Photograph showing the droplet generator microfluidic device assembled with the interface, connectors and fluidic tubing placed on a stereo microscope light stage.

Chip Surface Conditioning

The microfluidic device used was a Dolomite hydrophilic chip (3000436), which underwent surface conditioning** as reported in a peer reviewed journal. The reagents were purchased from Sigma-Aldrich and from Aquapel® respectively as described in the paper. This surface conditioning step allows the use of Dolomite's hydrophilic chip with Pico-Surf™ 2 (fluorinated oils) following the two separate protocols in separate tests, both with successful outcomes.

** ***Single-cell analysis and sorting using droplet-based microfluidics.*** *Linas Mazutis, John Gilbert, W Lloyd Ung, David A Weitz, Andrew D Griffiths & John A Heyman. Nature Protocols 8, 870–891 (2013)*

Reagent Preparation

1. Gelatin Particle Production

Droplet Fluid – Gelatin with 1st *Ab*

Two different solutions were prepared with the intention of running two different concentrations in order to assess its effect.

- Mix A (Low, 76 µg/ml)

  2 µl Mouse Serum in 0.5 ml gelatin mix (76 µg/ml)

  125 µl mTr + 123 µl water + 2 µl Mouse Serum + 250 µl 12.5 % Gelatin

- Mix B (High, 731 µg/ml)

  20 µl Mouse serum in 0.5 ml gelatin mix (731 µg/ml).

  125 µl mTr + 125 µl water + 20 µl Mouse Serum + 250 µl 12.5 % Gelatin

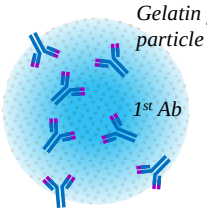
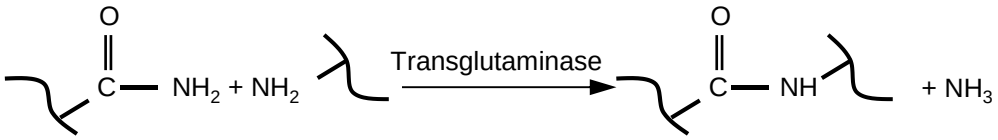


Illustration of a Gelatin Microparticle seeded with Mouse Reference Serum.

where, Transglutaminase (mTr) solution is as follows:

mTr = 1.5 ml 10x PBS + 2.5 ml water + 0.30 g Activa transglutaminase



This acyl-transfer enzyme catalyzes transamidation reactions that lead to the formation of N-ε-(γ-glutamyl) lysine crosslinks in proteins.

Carrier Fluid

FC-40/FC-70 mixture

Use of a fluorinated oil mixture with a higher viscosity

Mix = 2 ml Pico-Surf™ 2 (5% in FC-40) + 2 ml FC-70 + 1 ml FC-40

FC-70 has a 5-6 fold increased viscosity relative to FC-40

Pico-Surf™ 2 (surfactant), biocompatible in inert carrier oil (FC-40 or Novec7500).

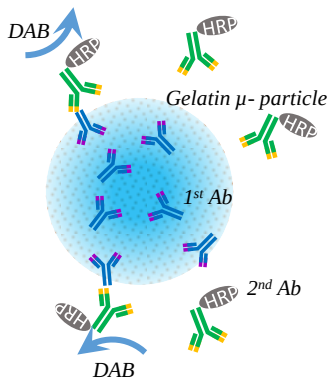
2. HRP-DAB Assay

Antibody	Type	Function	Product ID
1st *Ab*	Mouse Reference Serum	Primary protein/antibody for gelatin droplet incorporation.	Bethyl Laboratories, RS10-101 (contains 19 mg/ml IgG antibodies)
2nd *Ab*	Mouse IgG-Fc Fragment Antibody, HRP labelled	HRP-DAB Assay	Bethyl Laboratories, A90-131P

HRP detection assay (containing 2nd *Ab*)

Assay as described in manual for Mouse IgG ELISA Quantitation Set (Cat. no. E90-131, Bethyl Laboratories).

- Sample Conjugate Diluent (10 ml):
- 500 µl 1 M Tris pH 7.5
- 280 µl 5 M NaCl
- 1000 µl 10% BSA
- 500 µl 1% Tween 20
- 7.72 ml Sterile water
- Wash solution (10 ml):
- 500 µl 1 M Tris pH 7.5
- 280 µl 5 M NaCl
- 500 µl 1% Tween 20
- 8.72 ml Sterile water
- Reagent for detection of HRP coupled antibody (5 ml):
- DAB/Cobalt tablet + Urea Hydrogen Peroxide tablet (Sigma, D-0426)
- 5 ml sterile water (the reagent is used immediately after the tablets are dissolved in water)



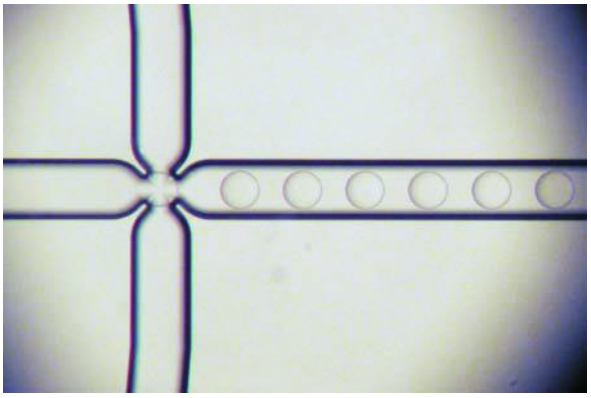
The interaction between the 1st Ab and the 2nd Ab results in the production of a deep blue color as depicted by the DAB signal.

Results

Droplet production

Droplets were produced by running the droplet system at the following conditions.

Droplet Pressure (mbar)	Droplet Flow Rate (µL/min)	Carrier Pressure (mbar)	Carrier Flow Rate (µL/min)
500	~ 10	1000	~ 25



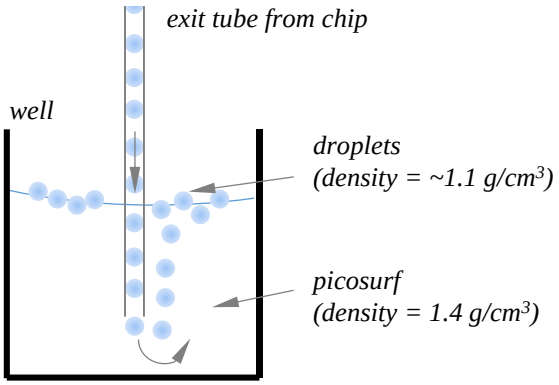
Prior to the color staining, all particles appear identical regardless of whether they contain the concentrated or dilute 1st Ab.

Droplets produced at the junction travel off-chip into the exit tubing as shown in an earlier schematic. The tubing is about 500 mm long and can be directed to a collection vessel. Here, a 96 well plate is used for collecting the sample. The tubing is steered by hand and sample is collected for a few minutes. The objective is to collect a small number of droplets so that they maintain a monolayer configuration in the well plate.

Droplet Harvesting

The hydrophobic nature of the well plate helps stabilize the droplets since the chip and tubing are also hydrophobic. Additionally, in order to keep the droplets away from the bottom and side walls of the well plate, extra FC-40 is manually added just prior to collecting sample. The FC-40 being of higher density than water helps the collected sample stay afloat on top.

Once collected in a well, the droplets continue to polymerize while they float atop the Pico-Surf™ 2. Once all sample is collected, the well plate is then placed in a humidity box in an incubator at 37°C in a



In a wellplate, the droplets continue to polymerize while they float atop the Pico-Surf™ 2. The well plate is then placed in a humidity box in an incubator at 37°C in a CO2 environment. The polymerization completes within a few minutes.

CO_2 environment. Extra Pico-Surf™ 2was added to each Well. The polymerization completes within a few minutes.

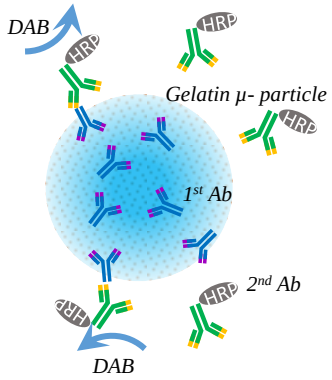
The next day, a small portion of solidified droplets were taken out of the well plate using a Micropipette. This was transferred to a fresh well plate. In this well plate, the droplets were carefully and sequentially washed with FC-40 (3× serial dilution and infranatant removal) to remove the Pico-Surf™ 2 molecules.

The sample was then ready for the HRP tests to be conducted at two different temperatures.

- Room temperature test – Droplets and oil was transferred to a 1.5 ml Eppendorf tube and stored at 4°C. Droplets were stored as a fluorinated oil mixture to avoid the loss of protein due to diffusion which would have occurred if the droplets had been stored as a water suspension. Before the HRP assay the droplets were resuspended in PBS buffer.
- Low temperature test – A portion of the droplets were used directly after resuspension, the rest were stored at -20°C for later use.

Room Temperature HRP Test

A suitable number of droplets of each type was transferred to a 24 well plate. Addition of 1x PBS and transfer of droplets to the water phase. Preparation of 3 wells for HRP reaction test. One well with low concentration droplets, one well with high concentration droplets and one well with both types of droplets. All liquid is removed from the droplets in the test wells (as much as possible). Addition of 500 µl HRP mix to all 3 wells.



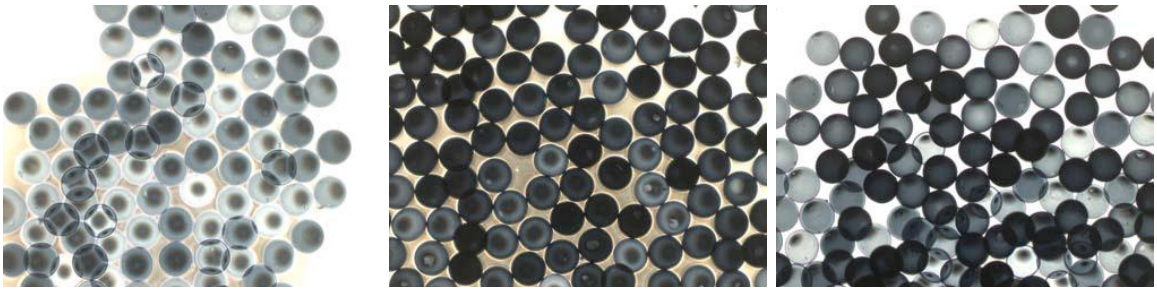
The interaction between the 1st Ab and the 2nd Ab results in the production of a deep blue color as depicted by the DAB signal.

- HRP mix = 2 ml Sample Conjugate Diluent + 2 µl Antibody 2 (HRP coupled)
- Incubation at room temperature: 10^{50} - 13^{00}.
- Droplets were washed 5 times with 500 µl Wash Solution.
- Preparation of DAB color reagent for HRP detection.
- Addition of 500 µl color reagent to all 3 wells.
- The reactions were started approximately at the same time and later on stopped at approximately the same time (to allow direct comparison of color development). The droplets developed a blue color at a reasonable speed, reactions stopped after a few minutes by removal of the staining solution and repeated wash with 1x PBS.

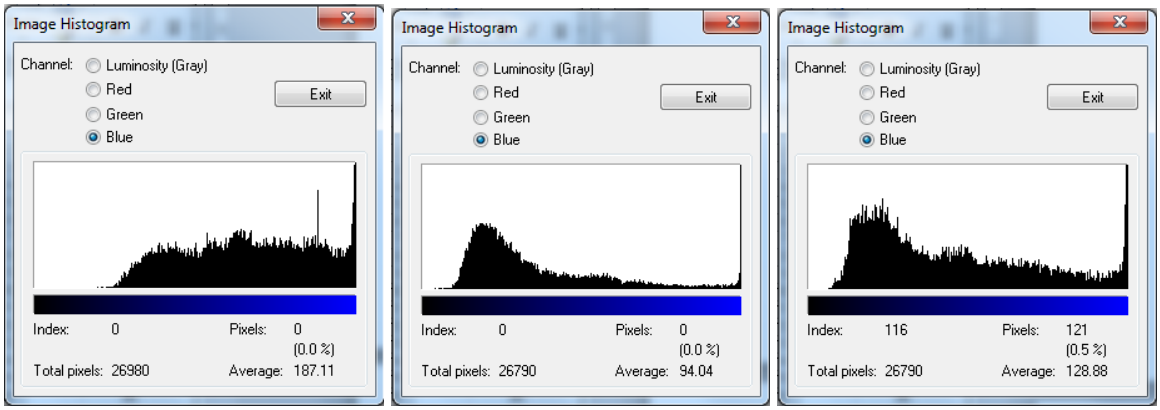
(1) Low *(2) High* *(3) Mix*

Gelatin particles collected in a well plate after HRP-DAB ready for visualization. The blue colored stain is visible to the naked eye as are the various grades of the blue indicating variable levels of DAB color reaction.

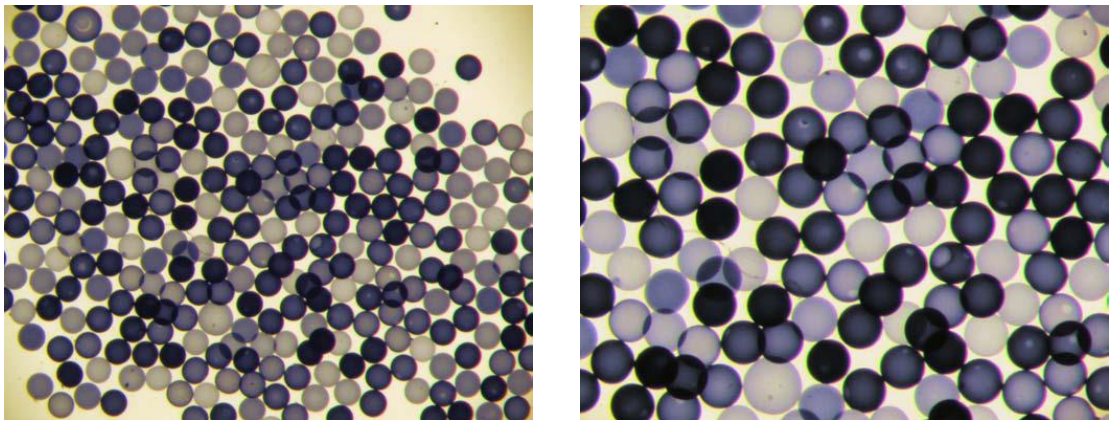
Microscopy of droplets using 4x objective of mixed droplets after HRP color reaction using a stereomicroscope (Nikon SMZ800). Pictures were taken using the same settings of light and camera parameters, this to allow direct comparison of the color intensities.



Left: Mix A: Low: 76 µg/ml. Middle: Mix B: High 731 µg/ml. Right: Mixed (Mix A + Mix B) 76 and 731 µg/ml.



Color intensity histograms of the blue channel. The left size of the scale indicates a dark color, whereas the right side of the scale indicates lighter hues of blue, tending to white. Left: Low concentration. Middle: High concentration. Right: Mix.



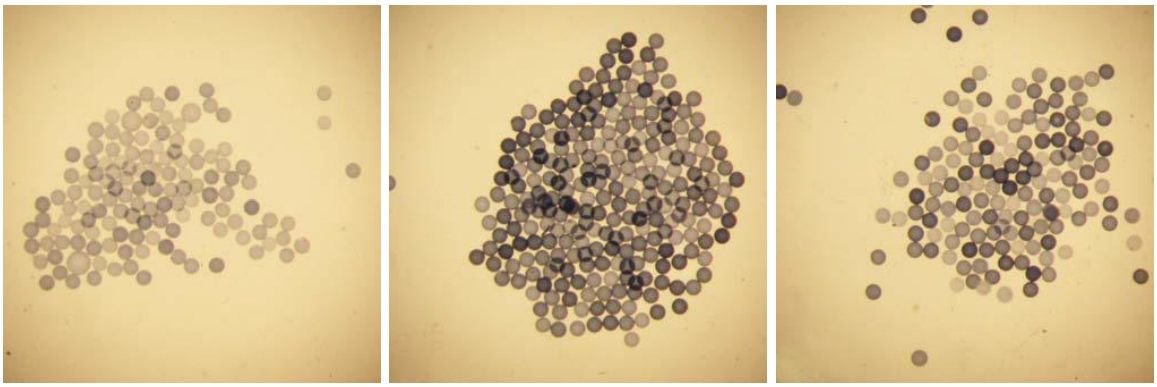
Mixed concentration. Left: Low Magnification. Right: High Magnification.

Low Temperature HRP Test

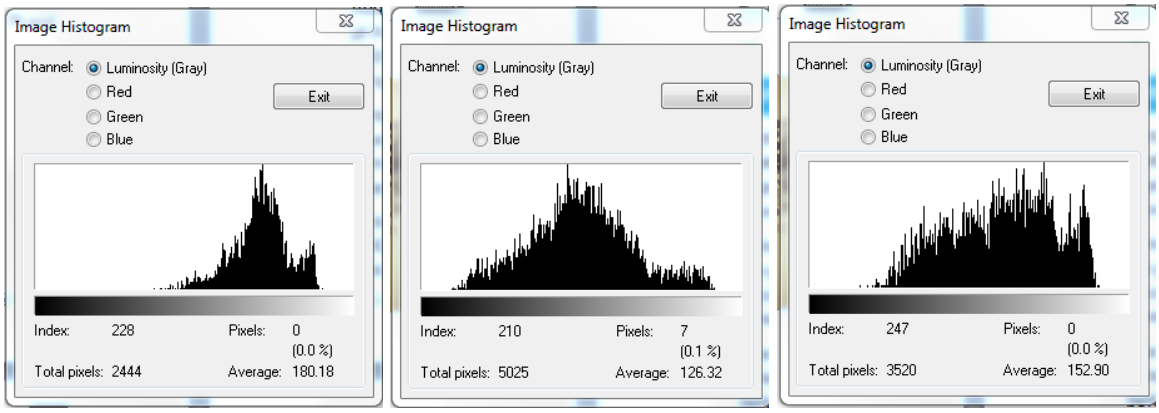
Repeated HRP assay using droplets stored at -20°C. This to test if droplets survive freezing and if the protein signal survive the storage procedure (is there a rapid loss due to diffusion) Preparation of 3 wells for HRP reaction test. One well with low concentration droplets, one well with high concentration droplets and one well with both types of droplets. All liquid is removed from the droplets in the test wells (as much as possible). Addition of 500 µl HRP mix to all 3 wells. HRP mix = 2 ml Sample Conjugate Diluent + 1 µl Antibody 2 (HRP coupled) Incubation at room temperature (in the dark): 1625 - 1655. OBS: Use of half the amount of detection antibody and half the incubation time as before. Droplets were washed 5 times with 500 µl Wash Solution.

Preparation of DAB color reagent for HRP detection. Addition of 500 µl color reagent to all 3 wells: 1720. The reactions were started at the same time and later on stopped at the same time. The droplets developed a blue color at a reasonable speed, reactions stopped after 5-10 minutes by removal of the staining solution and wash with 1x PBS.

Pictures of mixed droplets after HRP color reaction using a stereomicroscope (Nikon SMZ800). Pictures were taken using identical settings of light and of camera parameters. Also use of minimal and identical processing of the digital images.

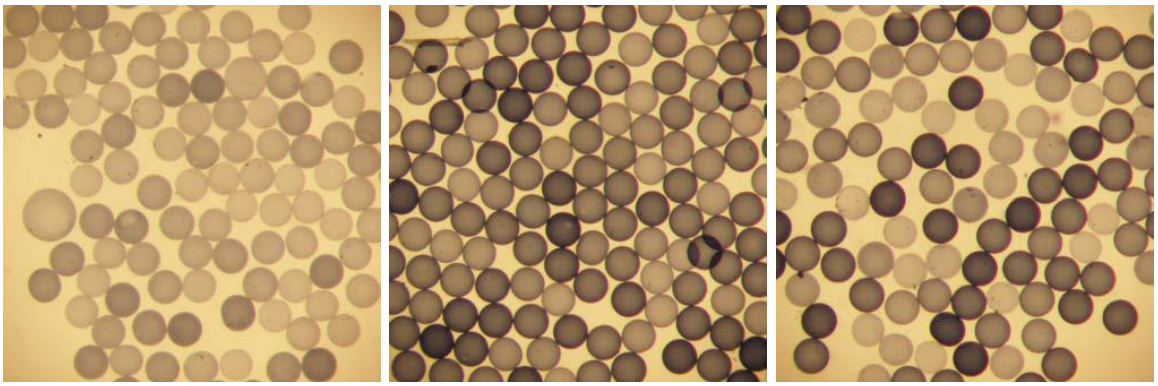


Left: Low 76 µg/ml. Middle: High 731 µg/ml. Right: Mix 76 or 731 µg/ml.



Color intensity histograms of the blue channel on a sub-population. The left size of the scale indicates a dark color, whereas the right side of the scale indicates lighter hues of blue, tending to white. Left: Low concentration. Middle: High concentration. Right: Mix.

Pictures of mixed droplets after HRP color reaction using a stereomicroscope (Nikon SMZ800). Pictures were taken using identical settings of light and of camera parameters. Also use of minimal and identical processing of the digital images.



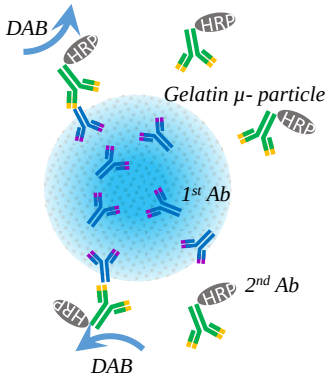
Left: Low 76 µg/ml. Middle: High 731 µg/ml. Right: Mix 76 or 731 µg/ml.

Conclusion

In this application note, we demonstrate a colorimetric based quantification of protein encapsulated in micrometer sized solid gelatin particles. The gelatin particles are manufactured using Dolomite's droplet system illustrating its application towards a slow release method for therapeutic proteins delivery using microfluidic technology. The microfluidic approach assisted with production of highly monodisperse droplets which were then converted to gel phase Gelatin, a hydrogel with high porosity and high water content.

A known quantity of Mouse Reference Serum protein was intentionally seeded in the particles. A second protein Mouse IgG-Fc Fragment Antibody, HRP labelled was used to detect the encapsulation of the first protein using a lab-standard HRP assay method. The tested Gelatin particles showed a positive color reaction (HRP assay) appearing different shades of blue.

Protein added to the gelatin before cross-linking is probably also exposed on the droplet surface, where the secondary antibody (HRP coupled) can detect it. This surface complex can survive the washing procedure, and it is not lost due to shear forces or diffusion in excessive amounts. Additionally, the protein macromolecules contained in the core of the hydrogel particles continue to diffuse out over time.



The interaction between the 1st Ab and the 2nd Ab results in the production of a deep blue color as depicted by the DAB signal.

Two different concentrations (low 76 µg/ml, high 731 µg/ml.) varying by 10X were used to demonstrate the quantification. Both the low and high protein concentrations resulted in variable droplet color intensities.

It is theorized that the small droplet size and hence extremely high surface to volume ratios lead to rapid diffusion. This leads to a broad peak distribution which is seen in the range of shades of blue. Additionally, it is unknown whether the shear stress acting on the droplets (surface) causes a lubrication layer that hinders contact between 1st *Ab* and 2nd *Ab*. It is

The HRP detection signal is clearly quantitative. The detection signal reflects the droplet protein concentration, and is shown to be able to detect a 10 fold difference. A mass transport based theory is presented to explain the broad distribution observed in the protein quantification. Storage and transport after storage at -20°C the droplets still showed a functional protein detection signal (HRP assay).

This study demonstrates the encapsulation and particle production method using a microfluidic approach. Further optimization will be required to control the release profiles, and these can simply be tweaked by changing the mechanical properties of the gel phase of which the Gelatin particle is composed (such as release profiles with specific proteins in a tissue environment).

While this method remains powerful for enabling protein delivery, it may be used interchangeably with suitable changes to adapt to drug delivery, as well as delivery of other important biochemical agents and macromolecules. Such methods may also be useful for tagging droplets for sorting applications, or for barcoding droplets for

identification. Overall, this study shows microfluidic synthetic techniques to have great potential for engineering and manufacturing delivery systems for therapeutic and diagnostic applications.

Acknowledgement

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The areas of stem cell biology and mammalian cell based protein manufacturing are developing fast. The ability for gentle cell sorting technologies and methods is expected to enable customers exploit unprecedented applications of cell biology in technology.

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Commercial Partner: Bioneer, Denmark (www.bioneer.dk/).

APPENDIX A: System Component List

Part No.	Part Description	#
3200177	Mitos P-Pump Remote Basic	2
3200178	Mitos P-Pump Remote Chamber 30	2
3200097	Mitos Flow Rate Sensor (30-1000µl/min)	1
3200098	Mitos Flow Rate Sensor (1 - 50µl/min)	1
3200200	Mitos Sensor Interface	2
3000024	Linear Connector 4-way	2
3000155	Chip Interface H	1
3000436	Droplet Junction Chip (190µm etch depth)	1
3000397	T- Connector ETFE	1
3200272	Flow Resistor Kit	1
3200063	FEP Tubing, 1/16" x 0.25mm, 10 metres	1
3200300	FEP tubing, 1/16" x 0.1mm, 10 metres	1
3200197	USB to RS232 Adaptor Cable	2
3200087	2-way In-line Valve	2
3200050	High Speed Camera and Microscope System	1
3200128/ 3200118	Mitos P-Pump Compressor 6bar	1
3200075	Advanced Droplet Starter Kit	1

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