Microgels as Artificial Cells in Modeling the Flow of Neutrophils in the Pulmonary Microcirculation

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract.

In this study the role of passive mechanism for deformation of neutrophils, namely the effect of mechanical properties, was studied using microgels as model system. Both alginate-poly(N-isopropylacrylamide) interpenetrating polymer network (IPN) microgels and agarose microgels were synthesized in microfluidic device.

The Young’s modulus and relaxation time of the IPN microgels were studied using atomic force microscopy equipped with a tipless cantilever. The lower limits of the elasticity found in this study were within the range of the elasticity reported for neutrophils.

Agarose microgels were also prepared with a range of elastic shear modulus similar to neutrophils, and their flow under constrained geometries was studied. The flow profiles of four agarose microgel samples in a microchannel containing a constriction were analyzed. It was found that the stiffness of the microgels affected their velocity before, in and after the constriction.
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—— 1.0 wt.% agarose microgels (G’=92 Pa), —— 1.5 wt.% agarose microgels
(G’=362 Pa) and —— 2.0 wt.% agarose microgels (G’=798 Pa).
Preface

This thesis has been organized as a manuscript that has been published in peer-reviewed scientific journal and unpublished results with critical comments and revision by Prof. E. Kumacheva. As identified by authorship, N. Raz designed and carried out experiments, data analysis and interpretation; and article writing as a first author. Prof. E. Kumacheva and corresponding collaborators provided critical comments and revision. The contributions of other authors are provided in detail below.

Chapter 3: Preparation of IPN microgels as model system in cell studies

The results in this chapter are mainly from a manuscript published in *Macromolecules*, 2010, ASAP by Neta Raz, James K. Li, Lindsey K. Fiddes, Ethan Tumarkin, Gilbert C. Walker and Eugenia Kumacheva.

**Contribution:** N. Raz contributed to the paper by designing and carrying out key experiments, data analysis and interpretation, and article writing. J. K. Li carried out AFM measurements, provided the IgorPro code for the calculation of mechanical properties of the microgels and revisions to the manuscript. L. K. Fiddes helped with the flow experiments of the microgels. E. Tumarking carried out some of the preliminary AFM work. G. C. Walker provided critical guidance and useful suggestions and discussions on the experimental design, and data analysis and interpretation.

Chapter 4: Correlation between the mechanical properties of microgels and their flow in confined geometries

The results in this chapter are from un-published result by Neta Raz, Lucy Chung, Lindsey K. Fiddes and E. Kumacheva (*in preparation*).
Contribution: N. Raz contributed to this chapter by carrying out key experiments, data analysis and interpretation. The elastic shear modulus of agarose hydrogels was measured by E. Kumacheva at Dr. Erika Eiser’s lab at the University of Cambridge. L. Chung and L. K. Fiddes are studying the effect of microgel initial velocity, constriction opening angle and microgel size on the microgels flow under constrained geometries. In later stages all results will be combined together to one publication.
Chapter 1
Introduction to Microfluidic Synthesis of Microgels and Microgels as Artificial Cells to Model the Flow of Cells in the Pulmonary Microcirculation

1.1 The lungs

The pulmonary circulation is a complex network of blood vessels in which gas exchange between the body and the air occurs.\(^1\) It contains 40-100 capillary segments with diameters ranging from 2 to 15 \(\mu\)m.\(^2\) While the total blood flow rate in the lungs is the same as in the systemic circulation, the pulmonary circulation contains about a quarter of the volume of the systemic blood circulation.\(^3\) Also, the pulmonary circulation differs from the systemic circulation and is unique for several reasons.\(^1,4\) For example, the pulmonary circulation is a low pressure and low resistance system. The maximal pressure in the pulmonary arteries is about 15 mmHg which is only one sixth of the pressure in systemic arteries. In addition, as opposed to other regional circulations which are set in parallel, the pulmonary circulation is a compulsory path for the whole blood.

In addition to their role in respiration, the lungs are an important site for host defense (the body protective response to a threat either by a physical barrier or by the immune system), since they receive all the venous blood and they have a very large surface area that is in contact with the external environment.\(^2\) One of the host defense mechanisms that is active in the lungs is the “marginated pool”. The marginated pool is the presence of neutrophils in the lungs in concentration 45-60 times larger than in a large blood vessel.\(^2\)
1.2 Neutrophils

Neutrophils, or in their full name, *neutrophilic polymorphonuclear leukocytes*, are the most abundant type of white blood cells. On average, their concentration in the blood is 40-65 % of all the white blood cells.\(^5\) Non-activated neutrophils are neutrophils that flow in the blood circulation. They are spherical and have an average diameter of 6-8 µm.\(^2\) Upon activation, by chemical or physical stimuli, neutrophils adhere to the endothelium and migrate into the tissue. While activated, neutrophils show a range of morphologies such as elongated, flattened shapes or contain pseudopods (temporary projections).

1.3 Flow of neutrophils in lung capillaries

The study of flow of blood cells in the cardiovascular system has great importance for understanding the response of the body to injuries and infections.\(^6\) In particular, much attention has been received to studies of flow of neutrophils in the pulmonary capillary network. As mentioned before, the diameter of neutrophils ranges from 6 to 8 µm, whereas the diameter of blood capillaries can be as small as 2 µm. Therefore, neutrophils are larger in diameter than about 40 % of the capillaries in the lungs.\(^7\) Thus, neutrophils are forced to deform in order to enter and flow through a capillary segment.

When comparing the flow of neutrophils to the flow of red blood cells in capillaries, it is seen that although the diameters of the two cells type are somewhat similar (red blood cells have the
neutrophils require longer time than erythrocytes to travel through the pulmonary capillary system. While red blood cells can flow through the lungs within several seconds, the flow of neutrophils can take more than 20 minutes. The longer transit time of neutrophils than of red blood cells in the lungs can be partially explained by the difference in the mechanical properties of the two cell types, since neutrophils are significantly more rigid than red blood cells. Therefore, the mechanical properties of neutrophils play an important role in their flow in the microcirculation. An in vivo study of flow of neutrophils in dog lungs showed that the transit time of neutrophils in the lungs was longer than the transit time of plasma and ranged from 2 s to 20 min. These long transit times were observed to originate in delays at discrete sites along the lungs circulation rather than in a low uniform velocity flow. A stop for time longer than 0.5 s in a capillary junction or a segment was found for 54% of the neutrophils in an in vivo study of dogs. This delay causes an increase in concentration of neutrophils in the lungs, as described earlier as the marginated pool, and an increase in pressure at the capillary segment entrance. In addition, a delay in the entrance of a capillary may lead to adherence of the cells to the endothelium that results in a hindrance of blood flow and is referred to as the leukocyte plugging phenomenon.

1.4 Mechanical properties of neutrophils

The mechanical properties of neutrophils have been studied by various techniques. The Young's modulus of non-activated neutrophils reported in the literature ranges from 200 to 2800 Pa. Under stimulation (either chemical or physical stimulation) neutrophils may become stiffer or softer depending on the nature of the stimulation. Roca-Cusachs et al. measured the elastic and
loss shear moduli of passive and stimulated rat neutrophils in AFM in frequencies, ranging from 0.1 to 102.4 Hz.\textsuperscript{14} The elastic and loss shear moduli were found to be in the range from \(~250\) to \(~708\) Pa and \(~80\) to \(~630\), respectively. Neutrophils, stimulated by adherence to glass surface showed a decrease in both the elastic and shear moduli. Wojcikiewicz \textit{et al.} found that while the Young's modulus of resting lymphocytes hybridoma (an hybrid cell of fused lymphocytes and cancer cell) was 1400 Pa, the modulus of chemically stimulated cells decreased to 300 Pa or increased up to 3000 Pa depending on the stimulus.\textsuperscript{16} Moreover, neutrophils' morphology, spreading area and motility were found to be depended on the stiffness of the substrate,\textsuperscript{17} and these factors are directly related to the mechanical properties of the cells.\textsuperscript{14} Measured by using an optical trap, the protrusional stiffness of the surface of human neutrophils was found to range from 0.06 to 0.11 pN/nm and was not depended on the receptor that was pulled.\textsuperscript{18} In addition, no dependence was observed between the temperature and the stiffness at room temperature and 37°C.

The viscoelasticity and stress relaxation of human neutrophils was extensively studied using a micropipette aspiration technique.\textsuperscript{11,19-21} The viscosity of neutrophils reported in the literature ranges from 6.5 to 89 Pa\cdot s depending on the model chosen to describe the cells.\textsuperscript{22} Typically, the models that are applied to describe neutrophils are the standard solid, Maxwell and Newtonian models. Yanai \textit{et al.} found that stress relaxation of neutrophils has a power law behavior in time and the exponent was measured to be \(~0.5\).\textsuperscript{23} The recovery of neutrophils after micropipette aspiration was depended on the time the cells were held in the pipette. In short holding times a viscoelastic behavior was observed while in long holding times the cells acted as a Newtonian liquid.\textsuperscript{21} Viscoelastic behavior of the cells was also proven in relaxation time experiments using
an optical trap. Direct evidences for the cells viscoelasticity was found in their relaxation time and hysteresis, and indirect evidence was found in the dependence of the protrusional stiffness on the loading force rates.

1.5 Effect of mechanical properties of neutrophils on their flow in the microcirculation

The effect of the mechanical properties of neutrophils on their flow in the microcirculation system is reflected by the long time it requires the cells to regain their shape after deformation and their stiffness. Due to their relatively long relaxation time, the shape restoration of neutrophils may take from tens of seconds to minutes. A study of the aspect ratio (the ratio between the cells major to minor axes) of neutrophils in the arterioles and venules showed that while neutrophils are nearly spherical in the arterioles (the aspect ratio of 94% of the neutrophils was smaller than 1.25), in the venules 53% of them had aspect ratio above 1.25, that is, they were elongated. The dimensions of the cells were not provided.

Moreover, the formation of pseudopods (temporary projections) is essential for emigration of leukocytes into the extravascular tissue. However, Gaehtgens suggested that pseudopods formation is hindered in leukocytes that are passively deformed (have an elongated shape due to geometrical constraint). According to this hypothesis, pseudopods formation is hindered as long as the cell is passively deformed. Thus, due to the long relaxation time of leukocytes the pseudopods formation can be hindered for long time after the cell exit the constriction.
Lastly, since leukocytes are less deformable than erythrocytes, leukocytes diameter during the flow in a capillary is larger than the diameter of erythrocytes. This difference in the cells size is associated with an increase in the resistance to blood flow. In addition, due to the lower velocity of white blood cells in the capillaries than the velocity of red blood cell, red blood cells are accumulating behind a white blood cell in the capillaries and form a train. This train formation causes an increase in the local resistance to flow.\textsuperscript{13}

### 1.6 Artificial cells

Studies of flow of cells are challenging for a number of reasons. For example, understanding the role of specific factors on cell behavior is difficult due to the large variability between cells of the same line. Also, an accurate variation of a particular single variable, e.g., the value of elastic modulus or the adhesion of cells to the substrate is sometimes impossible. Furthermore, cells have complex morphologies.\textsuperscript{24,25} Even when the same cell line is studied, the effect of the environment on cells has also an important role in \textit{in vitro} studies. This was demonstrated in neutrophils studies, when the stiffness of the substrate used affected the cells’ morphology, spreading area and motility.\textsuperscript{17}

For all the reasons listed above, studies conducted on model systems are sometimes preferred.\textsuperscript{24,26-28} The use of vesicles,\textsuperscript{25,26,29} beads,\textsuperscript{27} microgels\textsuperscript{30,31} or gels encapsulated in vesicles\textsuperscript{24,32,33} allowed control and consistent variation of their size, and surface and mechanical properties. For example, the study of vesicle and artificial cell membrane interactions was conducted mimic the process of exocytosis which involves fusion of a neurotransmitter-
containing vesicle with cell membrane. This artificial system allowed exclusion of the effect of membrane proteins on the exocytosis process. In a different study, cell membrane proteins were incorporated onto polystyrene beads as artificial cells to isolate the effect of specific proteins on cell-cell interactions.

1.7 Microfluidic reactors for the preparation of microgels

Microfluidics (MFs) involves the use of micro-scale channels for handling atto- to nano-liters of fluid. The ability to manipulate flows in MF device enables the production of bubbles and droplets in a carrier phase, which can serve as precursors in the production of polymer particles and foams. MF enables the generation of highly monodisperse droplets with controlled sizes, shapes and morphologies. The main geometries of MF devices that are used to generate droplets and bubbles include the flow-focusing, T-junction, co-flow and terrace designs.

1.7.1 Flow focusing devices

In a flow focusing MF device three streams are brought into contact in an orifice (Figure 1.1). The dispersed phase is introduced to the MF device through the middle stream (stream A in Figure 1.1) and the carrier phase is introduced to the device through the two outer streams (streams B, Figure 1.1). The carrier phase forces the inner phase into an orifice in which the latter breaks up into droplets. For a particular combination of liquids, the ratio between the flow rates of the dispersed and continuous phases and the dimension of the orifice determine the size and dispersity of the droplets.
1.7.2 T-junction devices

In T-junction device the dispersed phase (stream A in Figure 1.2) is introduced into the device perpendicular to the stream of the carrier phase (stream B, Figure 1.2).\textsuperscript{38,39} Droplets are formed due to the high shear forces imposed on the dispersed phase by the carrier phase at the junction.\textsuperscript{38} The ratio between the flow rates of the dispersed and continuous phases and the dimensions of the channels determine the size and dispersity of the droplets.\textsuperscript{39}

1.7.3 Co-flow devices

Figure 1.3 presents a schematic of the droplet generator part in a co-flow device.\textsuperscript{40-42} The dispersed phase (stream A in Figure 1.3) is injected into the stream of the continuous phase.
(stream B, Figure 1.3). Droplets formation is governed by two mechanisms: formation of droplets at the capillary tip (which is referred as the dripping mechanism) or the breakup of the stream after it exits the capillary (which is referred as the jetting mechanism). The flow rates of the continuous and dispersed phases and the dimension of the capillary determine the size of the formed droplets.42

![Diagram](image)

**Figure 1.3.** Schematic of the droplet generation part of co-flow device. The dispersed phase is introduced to the device through a capillary (stream A) that is injected into the carrier phase (stream B).

### 1.7.4 Terrace MF devices

In terrace MF devices (Figure 1.4) droplets are formed while they fall from the stream of the dispersed phase (stream A in Figure 1.4) into a well.43,44 The droplets are then carried by the continuous phase that flow perpendicular to the dispersed phase in the well. The droplets that are formed in terrace devices have a disk-like shape, however, due to the interfacial tension they transform into spheres.46 The rate of droplet formation depends on the flow rate of the dispersed phase46 and the size of the droplets is determined by the dimensions of the device.46
Figure 1.4. Schematic of the droplet generator part in a terrace device. The dispersed phase is broken to droplets while they fall from the edge of a step (stream A). The continuous phase flows in perpendicular to the droplets to carry them down the stream.

1.8 Flow studies in microchannels

1.8.1 Flow studies using synthetic particles

Studies of flow of particles in microchannels have a broad range of applications, such as modeling the flow of cells and biological systems, drug delivery, fundamental studies of flow of complex fluids and modeling the flow of particles in studies for the microelectronic manufacturing industry.

MF is advantageous for modeling biological systems in general, and the flow of blood cells, in particular, for several reasons. First, the flow in microchannels is laminar and the Reynold’s number can be easily adjusted to be close to the Reynold number of blood in blood vessels. Secondly, using a soft lithography method, microchannels can be easily produced with a variety of designs to mimic the blood circulation. Lastly, the internal structure and surface of the microchannel can be readily modified by various methods.
The effect of interactions between microgels and channel walls on their flow in constraint geometries was studied in microchannels containing a constriction. The electrostatic forces acting between the channel walls and the microgel surface affected the velocity of the microgels when they passed through the orifice. Receptor-ligand interactions between the surface of microgels and constriction walls were found to decrease with increase in the microgels velocity before the constriction. These receptor-ligand interactions were also affected by the rigidity of the microgels, as a lower velocity was observed in the constriction (relative to the microgels’ initial velocity) for the compliant microgels. Haghgooie et al. studied the effect of mechanical properties of polyethylene glycol microgels on their flow through confinements. As the rigidity of the microgels increased (controlled by increasing the cross-linking density of the microgels), they required higher pressures to deform and pass a constriction with width and height of about half of the microgel diameter. In addition, the effect of the microgel shape on its deformability was demonstrated by showing that S-shape microgels were the most flexible microgels and the particles became less flexible as their shape was altered to crosses, then rings and finally, disks which were found to be the stiffest shape tested. The difference in the flexibility of the four different shapes was attributed to the distribution of the mass in the volume of each of the microstructures.

1.8.2 Flow studies using living cells
Microchannels are also intensively used for in vitro study of flow of living cells. The transit time of red blood cells was studied in rectangular cross section microchannels with a cross section equivalent to a circle with diameter of 6 μm. In this study 2600 microchannels were connected in parallel, and the effect of the blood donors age, volume of red blood cells in the blood and donors diet on the flow of the cells was studied. Shelby et al. studied the flow of healthy and
malaria-infected erythrocytes (with a diameter and thickness of 8 and 2 μm, respectively) in microchannels fabricated in poly(dimethylsiloxane). The microchannels contained one confinement with a width ranging from 2 to 8 μm and height of 2 μm. The authors found that while the healthy cells and those in the early stages of the disease could pass all capillaries, cells in the later stages of the disease were not able to deform to the smallest capillaries. Some of the cells were not able to pass even through the 4 and 6 μm wide capillaries, however, healthy cells were still able to squeeze their way through a blockage of infected cells and the 6 μm wide capillary.

The flow of white blood cells in stenosed glass capillaries was studied to model the flow of blood in stenotic blood vessel caused by endothelial cell bulging. The flow of two samples of white blood cells through the capillary were studied. In the first sample whole blood sample was used as is while in the second sample the blood sample was separated to its components and then the white blood cells were re-dispersed in the plasma suspension. Analysis of the flow profiles of these two samples in the capillary revealed differences in the flow of the cells in these two cases. While in both cases the cells velocity increased just before and after the stenosis and sharply decreased in it, the increase in the velocity of the cells flowing in the whole blood sample was smaller than the re-dispersed sample. Also, only the cells in the whole blood sample were observed to stop in the stenosis.

1.9 Summary

The flow of neutrophils in the pulmonary circulation has great importance for understanding the function of the cells. Neutrophils are known to be relatively stiff blood cells and have long
relaxation times. Although the mechanical properties of neutrophils are widely studied, the effect of the mechanical properties on the cells flow in blood vessels has not been isolated when studying real cells in blood vessels. Therefore, a study of the flow of model cells in constraint geometries can contribute toward fundamental understanding of the behavior of neutrophils while they flow through blood capillaries.
1.10 References


Chapter 2
Materials and Methods

This chapter describes the materials and methods for preparation and characterization of microgels in microfluidic device and that were used in Chapter 3 and Chapter 4.

2.1 Materials

N-isopropylacrylamide (NIPAm), sodium alginate, \( N,N' \)-methylenbisacrylamide (BIS), undecanol, Span-80, mineral oil (viscosity of 30 cp), ammonium persulfate (APS), phosphate buffered saline (PBS, pH 7.4), mercaptoethylamine, Fluo-3, Ca\( \text{II} \), Rhodamine B and \( N,N,N',N' \)-tetramethylethylenediamine (TEMED) were purchased from Sigma Aldrich (Canada) and used without further purification. CaCl\( _2 \) was purchased from Fisher Scientific (Canada). Sylgard 184 Silicone elastomer kit which contained poly(dimethylsiloxane) (PDMS) prepolymer and crosslinker was purchased from Dow Corning Corp. (USA), SU-8 photoresist was purchased from MicroChem (USA) and Ultra low gelling agarose was obtained from SeaPrep (Switzerland).
2.2 Synthetic methods

2.2.1 Fabrication of microfluidic devices

Microfluidic flow-focusing\(^1\) and T-junction\(^2\) reactors were fabricated in PDMS using a soft-lithography procedure.\(^3\) The MF channel pattern was designed in Freehand software (Adobe Systems Inc., USA) and printed on a transparency. This transparency was then used as a mask for the preparation of the master from SU-8 50 photoresist on a silicon wafer. A PDMS negative replica of the master was prepared from a mixture of the prepolymer PDMS with the crosslinking agent in ratio of 10:1. The mixture was degassed for 40 min under vacuum, poured on the master and cured over-night at 75 °C. After curing, holes were drilled in the inlets and outlet of the sheet. The patterned PDMS sheet and a flat PDMS sheet were transferred to an oxygen plasma chamber (PDC-3XG, Harrick, USA) for 90 sec at 600 mTorr. The patterned PDMS sheet was sealed to the flat sheet immediately after the plasma treatment. Polyethylene tubing (Small Parts, USA) were placed in the drilled holes and sealed with epoxy glue.

2.2.2 Microfluidic synthesis of microgels

Alginate-PNIPAm microgels were synthesized in a flow-focusing droplet generator\(^2\) (Figure 1.1). The orifice width and height were 85 and 115 \(\mu\)m, respectively and droplet gelation occurred in an extension serpentine channel with a height of 115 \(\mu\)m and the length of 23.5 cm. Agarose microgels were prepared using a T-junction reactor\(^2\) (Figure 1.2). The widths of the channel at the junction were 30 and 150 \(\mu\)m for the agarose and continuous phase, respectively and the height of 110 \(\mu\)m. The liquids were supplied to the microfluidic device using syringes and polyethylene tubing. The flow rates were controlled by syringe pumps (Harvard Apparatus,
USA, PHD 2000 series). In general, the dispersed phase was an aqueous solution containing the materials to be gelled and the continuous phase was an oil solution containing a surfactant.

2.3 Characterization methods

2.3.1 Optical imaging

The microgels were imaged in an optical microscope (Olympus BX51, USA) equipped with a digital camera (CoolSnap ES, Photometrics, Roper Scientific, USA) and analyzed by ImagePro Plus 5 software (Media Cybernetics Inc., USA). In the measurements of polydispersity, the diameter of at least 50 microgels was measured in each sample and the distribution in the diameters was fitted to the Gaussian distribution.

2.3.2 Confocal microscopy

In order to image the alginate-PNIPAm microgels using confocal fluorescent microscopy, the microgels were stained with the fluorescent dye Fluo-3. The dye which is a tracer for Ca\(^{2+}\) ions was dissolved in the aqueous droplet phase and encapsulated in the microgels. The internal structure of the microgels in water was examined using confocal fluorescent microscope (Leica TCS SP2, Germany).

2.3.3 Swelling tests

The measurement of swelling ratio of the IPN microgels was done using macroscopic gel samples which were prepared in the same compositions as the microgels. For the hydrogel
preparation, a small Petri dish was filled with an aqueous solution of alginate, NIPAm, BIS and APS. The height of the solution was approximately 5 mm. Undecanol solution containing Ca\(^{2+}\) and TEMED was poured over the aqueous solution at a height of approximately 5 mm and left overnight at room temperature for gelation of alginate and PNIAPm. After 24 hrs, the undecanol solution was replaced with the post-treatment aqueous solution containing Ca\(^{2+}\), APS and TEMED for another 24 hrs. Then the hydrogels were washed with deionized water and transferred to PBS buffer in which swelling was tested. Approximately 4 g of the sample were weighted in the swollen and the dry states. The experiment was repeated three times for each sample. The swelling ratio was calculated as

\[
Q = \frac{m_s}{m_d} \cdot 100
\]  

(2.1)

where \(m_s\) and \(m_d\) are the masses of the swollen and dry hydrogel, respectively.

2.4 Mechanical properties measurements

2.4.1 Mechanical properties measurements in AFM

The Young’s modulus and the relaxation time of each sample of the alginate-PNIPAm microgels were measured using atomic force microscopy (AFM). The indentation experiment was done in a contact mode under fluid using an MFP-3D AFM (Asylum Research, USA). A 100 μm-wide tipless cantilever (Nano World, USA) was used with a nominal spring constant value of 0.03 N/m (the spring constant was more accurately determined by the thermal noise method\(^4\)). To immobilize microgels on the substrate and ensure that they remain under the cantilever during the indentation measurement, the particles were applied to a gold-coated quartz substrate (Ssens
B.V., Hengelo, The Netherlands) coated with a self assembled monolayer of mercaptoethyamine.\textsuperscript{5} Due to the presence of deprotonated carboxylic groups in alginate, the microgels carried a negative charge and were attracted to the positively charged substrate. The gold substrates were prepared by immersing them in piranha solution for 15 min, rinsing them with excess deionized water, and immersing them in a 1.0 mM mercaptoethylamine aqueous solution for at least 2 hrs. Finally the substrates were washed again with excess deionized water.

All AFM measurements were carried out in PBS buffer solution and the indentation experiments were performed for at least three microgel particles in each sample. Ten force curves were collected for each microgel. A typical force-time curve acquired in an indentation experiment is shown in Figure 2.1. First, a loading force was applied to compress a microgel particle until a setpoint of 1.5 – 2 nN was reached, as shown in the “compression” region. Then, in the “dwell” step the driving piezo was halted for 1-2 s, and during this time, a decay of the force, $F$, occurred, due to the relaxation of the stress on the microgel particle. After the dwell, the cantilever was retracted from the microgel (the “retraction” step).
The force-distance indentation results were fitted to the following equation using IgorPro software:\(^6\)

\[
\alpha = (3\pi)^{2/3} P^{2/3} \left( \frac{1-\sigma^2}{E} \right)^{2/3} \left( \frac{1}{D} \right)^{1/3}
\]  

(2.2)

where \(\alpha\) is the indentation distance, \(P\) is the force (pN), \(\sigma\) is the Poisson’s ratio (assumed to be 0.5), \(E\) is the Young’s modulus (Pa), and \(D\) is the microgel diameter (\(\mu\)m)

The dwell portion of the force vs. time curves was fitted to the Kohlrausch-Williams-Watts function (stretched exponential function) using IgorPro software.\(^7\)
where $F_r$ is the amplitude of the relaxation force (nN), $F_\infty$ is the force after a length of time much longer than the mean relaxation time, $\tau$, and $\beta$ is the stretching exponent. In cases where the system cannot be fitted to a single exponential decay, the stretching exponent, $\beta$, gives a measure of the distribution of the relaxation times.

### 2.4.2 Mechanical properties measurements using a rheometer

The elastic shear modulus, $G'$, of macroscopic agarose samples was determined in a stress-controlled rheometer (Physica MCR501, Anton Paar). The plate-plate geometry (with a diameter of 50 mm) was equipped with a true gap sensor, to maintain a constant gap of 50 mm throughout the measured temperature window of 4°C to 80°C. Furthermore a thermostated cap was used to avoid any temperature-gradients during measurement.

The elastic shear modulus was measured by frequency sweeps. Using the same geometry (granting a constant shear stress throughout the sample), 1 Pa stress amplitude was applied, which was within the linear regime in the gelled samples, varying the angular frequency from 0.1Hz to 50 Hz. Control experiments were done with lower stress amplitudes and also by applying a strain amplitude of 1 %, to make sure that the measurements capture the true viscoelastic response. In the gel phase, samples with agarose concentrations above 1.0 w%
showed a reproducible plateau in $G'$ for frequencies between 0.1 and 10 Hz. The 0.75 w% gels attained their plateau region at low temperatures only after 10 to 20 minutes.

2.5 Setup of experiments of flow of microgels under confined geometries

2.5.1 Microfluidic device

The flow of microgels in microchannels was studied in a microfluidic device fabricated in PDMS using a soft lithography method. Figure 2.2a shows a typical design of the microchannel used in these studies. The device contained a single straight channel with a length and height of 1.2 cm and 150 μm, respectively, and an orifice of 450 μm long at a distance of 1.0 cm from the channel’s inlet. The widths of the channel at the channel-at-large and the constriction were 160 and 62 μm, respectively.

The rectangular cross-section of the microchannel was transformed to a circular cross-section. A solution of 50 wt.% of PDMS in hexanes was injected into the rectangular cross-section channel at room temperature. Next, a nitrogen gas stream in pressure of 7 psi was flowed through the channel for 2 min at room temperature to template the circular cross-section and 10 more minutes at 100 °C to cure the channel. Finally the device was cooled down to room temperature and washed with ethanol and deionized water. After modification, the diameter of the microchannel in the channel-at-large and the constriction was 150 and 50 μm, respectively.
Figure 2.2. Schematic of the microchannel at the constriction part. a) rectangular cross-section microchannel. W1=160 μm, W2=62 μm, L1=450 μm, and θ=35°. b) modified microchannel to a circular cross-section. Black solid lines represent the original outlines of the microchannel; blue dash lines represent the modified outlines of the microchannel. D1=150 μm, D2=50 μm, L1=450 μm, θ1=35° and θ2=12°.

2.5.2 Flow of microgels through a microchannel containing a constriction

A dilute dispersion of microgels was transferred into 1.0 mL syringe (Henke Sass Wolf, Germany) the delivered to the microfluidic device through polyethylene tubing (Small Parts, USA) by a syringe pump (Harvard Apparatus, PHD 2000 series, USA). The flow rates used ranged from 0.1 to 0.18 mL/hr, depending on the sample tested. The flow of the microgels through the microchannel was viewed in a microscope (Olympus BX51, USA) and captured using a digital high speed camera (300 frames per seconds) (Casio EX-F1). Video clips of the microgels flowing in a 1.5 mm long segment of the channel with the constriction in the middle of the segment were transferred to a computer and the velocity of the microgels in each part of the channel was calculated. For each microgel sample tested at least 5 video clips were recorded. The video clips were then saved as still images and the position of the microgel in at least 30 images were analyzed.
2.6 References

Chapter 3
Preparation of IPN Microgels as Model System in Cell Studies


3.1 Introduction

Hydrogels are gels made of hydrophilic crosslinked polymers that are swelled in water.\textsuperscript{1,2} In many cases, the water content in hydrogels and their softness are similar to those of natural tissue. Thus, hydrogels are often used in pharmaceutical and medical applications, e.g. soft contact lenses, artificial skin, artificial muscles and wound and burn dressing.\textsuperscript{3} If hydrogels are mechanically weak,\textsuperscript{1,4} an improvement in their strength can be achieved by forming double-network hydrogels.\textsuperscript{1} The improvement in the mechanical strength of the double network hydrogel comes from a combination of first, highly crosslinked polymer with high Young’s modulus and a second loosely crosslinked or non-crosslinked polymer.\textsuperscript{4} The second polymer prevents the whole hydrogel from cracking by dissipating the fracture energy of the first polymer.

Double network hydrogels have a structure of interpenetrating polymer networks (IPNs). In IPNs, at least two polymer networks are combined together during their synthesis in such a way that one of the polymers is more cross-linked than the other.\textsuperscript{5,6} Although a molecular interpenetration in the network is desired, generally, IPN hydrogels are made of immiscible
polymers that phase separate during the synthesis. Nevertheless, these polymer networks are held together by permanent entanglements. The type of morphology an IPN system, that is, a partial IPN vs. a phase separated IPN, largely determine their mechanical properties.\textsuperscript{7} Partial IPNs are made of partially mixed polymers that are compatible with each other. Thus, these systems show one major relaxation maximum, e.g. one glass transition temperature. On the other hand, phase separated IPNs show two relaxation transitions, e.g. two glass transition temperatures. These relaxation transitions become prominent in the dependence of the mechanical properties on the transition temperatures which are shifted along the temperature scale.

Here we report the synthesis of double network polymer alginate-poly(\(N\)-isopropylacrylamide) microgels using microfluidic (MF) synthesis.\textsuperscript{1,8} Our method included MF generation of droplets of the solution containing a mixture of sodium alginate (to generate physically crosslinked first network) and NIPAm (to form chemically crosslinked second network), followed by the gelation of the precursor droplets. Sodium alginate was gelled by crosslinking the carboxylic groups with Ca\textsuperscript{2+} ions (Figure 3.1a) diffusing in the droplets from the continuous phase.\textsuperscript{9} Poly(\(N\)-isopropylacrylamide) (PNIPAm) was synthesized by polymerizing NIPAm (Figure 3.1b) and chemically crosslinked using \(N,N'\)-methylenebisacrylamide (BIS).

The use of MFs enabled precise control of the microgel size and structure.\textsuperscript{9-12} The dimensions of the microgels were tuned to be 100 ± 10 \(\mu\)m, in order to make them suitable for flow studies in MF channels. The variation in the mechanical properties of the microgels was achieved by
varying the ratio between the concentration of NIPAm and sodium alginate in the precursor droplets and the degree of crosslinking of the PNIPAm.
Figure 3.1. Reactions leading to the formation of gels with IPN structure. (a) crosslinking reaction of sodium alginate by calcium ions;\textsuperscript{13} (b) polymerization of NIPAm monomer to PNIPAm and cross-linking of PNIPAm by BIS.

3.2 Results and discussions

3.2.1 MF Synthesis of IPN microgels

The IPN microgels contained two networks; sodium alginate and PNIPAm. The synthesis was performed in a MF reactor, which included a droplet generator and an extension microchannel for droplet gelation. Figure 3.2 shows the schematic of the MF reactor, which contained two parts. The flow-focusing droplet generator\textsuperscript{14} had an orifice width and height of 85 and 115 \( \mu \)m, respectively. Droplet gelation occurred in an extension serpentine channel with a height of 115 \( \mu \)m and the length of 23.5 cm. Both chemical and physical gelation were achieved using the external gelation method reported by Zhang et al.\textsuperscript{9,11} and modified for the formation of IPN microgels. As shown in Figure 3.2, the aqueous droplet phase and undecanol continuous phase were introduced in the central and side microchannels of the reactor. The undecanol solution contained 0.25 wt. \% of CaI\textsubscript{2}, 1.0 wt. \% of \( N,N,N^{'},N^{''} \)-tetramethylethylenediamine (TEMED), and
2.0 wt. % of a non-ionic surfactant Span-80. The aqueous phase contained 0.1 wt. % of the redox initiator ammonium persulfate (APS) and varying amounts of sodium alginate, NIPAm, and BIS. The recipes used for the generation of microgels are shown in Table 3.1.

**Figure 3.2.** Schematic of the MF reactor for the synthesis of alginate-PNIPAm IPN microgels. TEMED and CaI\(_2\) are dissolved in the continuous phase (undecanol), which is supplied to the MF reactor from streams B. Sodium alginate, NIPAm, BIS and APS are dissolved in the aqueous droplet phase, which is introduced in the central channel (stream A). Following emulsification, TEMED and Ca\(^{2+}\) ions diffuse into the droplets and trigger polymerization of NIPAm and crosslinking of alginate, respectively.
Table 3.1. Recipes used for the preparation of IPN microgels

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content of sodium alginate* (wt. %)</th>
<th>Content of NIPAM* (wt. %)</th>
<th>Content of BIS* (wt. %)</th>
<th>Content of water* (wt. %)</th>
<th>Mean diameter of microgels (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1.0</td>
<td>1.0</td>
<td>10.0</td>
<td>97.8</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>A2, B2, C2</td>
<td>1.0</td>
<td>4.0</td>
<td>10.0</td>
<td>94.5</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>B1</td>
<td>1.0</td>
<td>4.0</td>
<td>1.0</td>
<td>94.9</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>C1</td>
<td>0.5</td>
<td>4.0</td>
<td>10.0</td>
<td>95.0</td>
<td>138 ± 8.6</td>
</tr>
<tr>
<td>C3</td>
<td>1.5</td>
<td>4.0</td>
<td>10.0</td>
<td>93.0</td>
<td>98 ± 4.6</td>
</tr>
<tr>
<td>C4</td>
<td>2.0</td>
<td>4.0</td>
<td>10.0</td>
<td>93.5</td>
<td>103 ± 5.5</td>
</tr>
</tbody>
</table>

* Concentrations of reagents in Table 3.1 are given for the droplet phase feed solutions. The continuous phase contained 0.25 wt.% CaI\(_2\), 1 wt.% TEMED and 2 wt.% Span-80 in undecanol.

As the aqueous droplets moved in the downstream channel, Ca\(^{2+}\) ions diffused from the continuous phase into the droplets and crosslinked alginate to form the first network as shown in the reaction scheme in Figure 3.1.\(^{13}\)

The second network was formed in parallel with the first one by the diffusion of TEMED into the droplets. TEMED accelerated the formation of radicals by APS and sped up the initiation of the polymerization reaction of NIPAm and the crosslinking of PNIPAm by BIS\(^{15}\) (Figure 3.2). The microgels were collected at the outlet of the MF reactor and transferred to an aqueous post-gelation solution containing 2.0 wt. % CaCl\(_2\), 0.1 wt. % of APS and 0.5 wt. % of TEMED for
overnight incubation. After the post-gelation step, the microgels were washed with deionized water and transferred to phosphate buffered saline (PBS, pH 7.4).

The IPN microgels produced in the MF reactor were divided into three series, in order to study the effect of each component (sodium alginate, NIPAm and BIS) on the mechanical properties of microgels (Table 3.1). In Series A the amount of NIPAm in the droplet phase was varied from 1.0 to 4.0 wt. %, while maintaining the concentrations of alginate and the crosslinking agent BIS constant. In Series B the concentration of BIS in the droplet phase was increased from 1.0 to 10.0 wt. % (the concentration of NIPAm and alginate were kept constant). In Series C the concentration of sodium alginate in the droplet phase was varied from 0.5 to 2.0 wt. % (the concentrations of NIPAm and BIS were kept constant).

To obtain microgels with dimensions suitable for the AFM measurements, the diameter of the IPN microgels prepared was tuned to be $100 \pm 10 \, \mu m$. This was achieved by tuning the flow rate of the droplet phase to be in the range from 0.05 to 0.1 mL/hr and the flow rate of the continuous phase to be in the range from 1.5 to 3.0 mL/hr. According to the rate-of-flow-breakup mode, smaller droplets are formed as the ratio between the flow rates of the continuous and droplet phases increases because of increased shear stress imposed on the droplet phase. Also, for the same reason, an increase in the viscosity of the droplet phase causes an increase in the droplet size for a given flow rate ratio of the continuous-to-droplet-phase. Therefore, a higher flow rate ratio of the continuous-to-droplet-phase was used for compositions of the droplet phase with higher viscosity.
3.2.2 Characterization of IPN microgels

3.2.2.1 Optical and confocal microscopy

An optical microscopy image of the microgel particles in PBS is shown in Figure 3.3a. The microgels were spherical and nearly transparent, due to the high water content. The narrow size distribution of the microgels that was achieved in the MF synthesis was preserved after the postgelation, washing and transfer to PBS buffer steps, as can be seen in the typical size distribution of the microgels (Figure 3.3b). The relative standard deviation in the diameters of the microgels in PBS was in the range of 4.7 to 13%.

![Image](a)

**Figure 3.3.** (a) Optical microscopy image of the microgels prepared from solution A1 (Table 3.1) and transferred to the PBS buffer. Scale bar 100 μm. (b) Gaussian fit to the size distribution of microgels prepared from Sample A1 (Table 3.1) and transferred to the PBS buffer.

The internal structure of the microgels was examined using fluorescence confocal microscopy. Figure 3.4 represents a characteristic confocal microscopy image of an individual microgel
particle stained with Fluo-3 dye. Since the fluorescence intensity of the dye is enhanced in the presence of Ca\textsuperscript{2+} ions, and since Ca\textsuperscript{2+} is the crosslinker of alginate, the fluorescence in the image was related to the presence of alginate in the microgel. Figure 3.4 shows a uniform distribution of fluorescence intensity throughout the particle, suggesting a homogeneous distribution of the crosslinked alginate in the microgel, with no significant macroscopic phase separation within the imaging resolution. The bright ring observed at the circumference of the microgel could be caused by the higher concentration of Ca\textsuperscript{2+} ions on the microgel surface, as well as by the optical effect.

![Confocal fluorescence microscopy image of the microgel particle prepared from Sample A2 (Table 3.1). Scale bar is 20 µm.](image)

**Figure 3.4.** Confocal fluorescence microscopy image of the microgel particle prepared from Sample A2 (Table 3.1). Scale bar is 20 µm.

### 3.2.2.2 Swelling experiments

The effect of the sample composition on the swelling ratio is shown in Figure 3.5 and Table 3.2. The macroscopic gels were prepared in a Petri dish by diffusion of Ca\textsuperscript{2+} and TEMED from a
solution in undecanol which was placed on top of the aqueous solution of alginate, NIPAm, BIS and APS (Chapter 2). The degree of swelling of the gel, \( Q \), immersed in the buffer solution increased with decreasing concentration of alginate and NIPAm in the samples and decreasing degree of cross-linking of PNIPAm (which was controlled by the concentration of BIS). A proposed explanation to these effects is that a polymer network is swelled until the solvent’s chemical potentials inside and outside the network become equal. When the network is crosslinked, the swelling is limited and balanced by the elastic forces arising from the stretched chains.\(^{17}\) Thus, the increase in the concentration of BIS in the samples decreased the degree of their swelling. In the case of increase in the concentration of alginate and PNIPAm in the samples, the networks were more entangled, \( i.e. \) their physical degree of crosslinking increased, and a similar effect to the increase BIS concentration was observed.

**Figure 3.5.** Effect of alginate concentration in the aqueous solution on the degree of swelling of the hydrogels. All aqueous solutions contained 4.0 wt. % NIPAm and 10.0 wt. % BIS (of NIPAm) in addition to alginate.
Table 3.2. Swelling ratio of samples A1, B1 and A2 and B2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Swelling ratio, Q (10^3 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>7.28 ± 0.49</td>
</tr>
<tr>
<td>B1</td>
<td>3.46 ± 0.11</td>
</tr>
<tr>
<td>A2 and B2</td>
<td>2.57 ± 0.04</td>
</tr>
</tbody>
</table>

3.2.2.3 Mechanical properties

The Young’s modulus and the relaxation time of the microgels were characterized in AFM experiments. Since the use of tipless cantilever is not typical in this kind of experiments, two notes are in order. First, the use of a cantilever with dimensions similar to those of the microgels enabled for indentation tests without pocking the microgels. The large cantilever also served as a better probe for measuring the mechanical properties of the entire microgel, as opposed to a conical tip, that probes the surface locally in the area of approximately about hundreds of square nanometers. Another widely used solution for the problems encountered with a use of conical tip is the use of microbead attached to the AFM tip as a probe. In these cases the Hertz model is usually used to calculate the elasticity of the sample. However, it should be noted that corrections to the Hertz model should be implemented to overcome the errors that arise from the use of this model.18 Second, the use of the tipless cantilever could lead to uncertainty in its alignment with respect to microgel, however in our work, for each microgel series, the deviation
in Young’s modulus did not exceed 19%, suggesting that the effect of misalignment did not dominate the experiments.

The first attempts to measure the mechanical properties of the microgels were conducted using mica as a substrate. In these experiments, the microgels tended to roll away from the cantilever probe and the results of measurements were irreproducible. Therefore, we printed a grid on a transparent sheet, in order to hold the microgels in a specific location and to prevent their movement during the indentation experiments. The grid had mesh with gaps of 100 μm and ridges height of about 5 μm. Figure 3.6 shows a droplet of microgel dispersion (Sample C3) on a grid printed on a transparent sheet. When the grid substrate was tested under the AFM setup, it was found that the ridges were too shallow and the microgels rolled over them during the measurement. In the third attempt, we used a gold substrate coated with mercaptoethylamine. Our assumption was that the microgels will be immobilized on the surface due to the attraction between the cationic groups of the mercaptoethylamine coated on the gold substrate and the negatively charged carboxylic groups in the microgels. The details of surface modification are provided in Chapter 2.
Figure 3.6. Microgel sample (C3) (see Table 3.1) applied to a grid printed on a transparent sheet. The yellow circle highlights a transparent microgel particle that seats between the grid ridges. Scale bar: 100 μm.

The mechanical properties of the microgels were studied as a function of their compositions. We examined two important mechanical properties of the microgels: their Young’s modulus and relaxation time. The details of the calculations of the Young’s modulus and relaxation time are described in Chapter 2.

Figure 3.7 shows the variation in Young’s modulus and relaxation time of the microgel samples in series C. Alginate concentration in the feed solution had a significant effect on the mechanical properties of the microgels. As the concentration of alginate in the feed solution was increased from 0.5 to 2.0 wt. %, the Young’s modulus increased from 1.86 to 29.0 kPa, that is, the microgels became stiffer (Figure 3.7a) and the relaxation time of the microgels (examined in stress relaxation experiments), was reduced from 305 to 16 ms (Figure 3.7b). The increase in the
concentration of alginate in the microgels increased the number of entanglement points in the polymer and the system relaxed faster. This relaxation can occur through the “thirion relaxation” mechanism,\textsuperscript{19} in which, reversible relaxation of physical cross-links or entanglements take place through reptation of polymer chains. Since the chains that are in constant reptation motion they can relaxed along their own contour.\textsuperscript{20}

![Graphs showing variation in Young’s modulus and relaxation time](image)

**Figure 3.7.** Variation in Young’s modulus (a) and relaxation time (b) of the microgels plotted as a function of the concentration of sodium alginate in the droplet phase. All samples contained 4.0 wt. % NIPAm and 10.0 wt. % BIS (vs NIPAm) in the feed solution and were tested at room temperature.

Since the microgels described in this chapter were prepared as a model system for cell studies, we attempted to tune the elasticity of the microgels to be similar to the elasticity of cells. The broad range of Young’s modulus of the microgels reported here is within the range of Young’s modulus reported in the literature for living cells (up to 100 kPa\textsuperscript{21}). In particular, the lower limit of the concentration of alginate yielded microgels with Young's modulus values of 1.86 ± 0.23
43 kPa, which is within the range of Young’s modulus reported in the literature for neutrophils. Further reduction in the Young’s modulus can be achieved in experiments conducted at 37 °C. However, because of the lower critical solution temperature of PNIPAm is approximately 32 °C, in samples that contain high concentrations of PNIPAm the opposite effect might be observed.

The effect of concentration of PNIPAm and of the degree of crosslinking in the microgels on the mechanical properties of the microgels was expected to be similar to the effect of alginate concentration. Table 3.3 shows the Young’s modulus and the relaxation time for the microgels with varying concentrations of NIPAm and BIS. Comparison of the properties of samples A1 and A2 showed a weak effect of the concentration of PNIPAm on their values of Young’s moduli, $E$, and relaxation time, $\tau$. Likewise, the Young’s moduli of samples B1 and B2 were relatively close. The weak effect of the concentration and degree of crosslinking of PNIPAm on the Young's modulus and relaxation time could originate from the partition of NIPAm between the droplet and continuous phases in the MF reactor, thereby reducing the concentration of this monomer in the microgel. Yet, the variation in the concentration of PNIPAm and its degree of crosslinking can serve as a tool for fine tuning of the mechanical properties of the microgels.
### Table 3.3. Young’s modulus and relaxation time of samples A1, B1 and A2 and B2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young’s modulus, $E$ (kPa)</th>
<th>Relaxation time, $\tau$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>9.9 ± 1.6</td>
<td>64.2 ± 16.1</td>
</tr>
<tr>
<td>B1</td>
<td>11.7 ± 1.4</td>
<td>46.3 ± 28.6</td>
</tr>
<tr>
<td>A2 and B2</td>
<td>11.4 ± 2.0</td>
<td>69.1 ± 50.4</td>
</tr>
</tbody>
</table>

* Samples A1 and A2 reflect the effect of PNIPAm concentration in the microgels.

Samples B1 and B2 reflect the effect of degree of crosslinking of PNIPAm in the microgels (Table 3.1)

As part of the calculation of the mean relaxation time of the microgels, the stretching exponent, $\beta$, was also calculated using Equation 2.3. In cases where the system cannot be fitted to a single exponential decay, the stretching exponent, $\beta$, gives a measure of the distribution of the relaxation times in the system.\(^{25}\) For example, in all samples the stretching exponent varied from 0.4 to 0.6 as summarized in Table 3.4. Interestingly, The weak power law dependence was in agreement with the trends observed for other biological systems such as hepatoma cells and fibroblast cells\(^{25,26}\) suggesting that the IPN microgels showed a cell-like-behavior.
Table 3.4. The stretching exponent, $\beta$, of the microgel samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stretching exponent, $\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td>C2, A2 and B2</td>
<td>0.59 ± 0.09</td>
</tr>
<tr>
<td>B1</td>
<td>0.57 ± 0.17</td>
</tr>
<tr>
<td>C1</td>
<td>0.53 ± 0.09</td>
</tr>
<tr>
<td>C3</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>C4</td>
<td>0.58 ± 0.25</td>
</tr>
</tbody>
</table>

* Samples A1 and A2 reflect the effect of PNIPAm concentration in the microgels.

Samples B1 and B2 reflect the effect of degree of crosslinking of PNIPAm in the microgels.

Samples C1-C4 reflect the effect of concentration of alginate in the microgels (Table 3.1).

3.2.2.4 Flow of microgels through microchannels

The application of the microgels as a model system for studies of flow of cells in blood capillaries was examined in microfluidics experiments. The microgel particles (Series C) were introduced in a microfluidic channel with a constriction, imitating flow in confinement in blood vessels. The rectangular cross-section of the microchannel was modified to a circular one to mimic better a circular cross-section of microvascular vessels.27 The diameter of the channel-at-large and the constriction were 180 $\mu$m and 50 $\mu$m, respectively (Chapter 2). A snapshot picture of a typical flow experiment conducted for sample C3 (Table 3.1) is shown in Figure 3.8. The microgels flowed through the channel-at-large until they reached the constriction entrance where they stopped, in order to deform to the required size to enter the constriction. The extent of deformation in our experiments was to about 50 % of the diameter of the microgels. However, for a given geometry of microchannel we can achieve different extent of deformations by tuning...
the size of the microgels. After deformation, the motion of the microgels through the constriction continued and they entered the channel-at-large at the exit of the constriction.

![Image](image_url)

**Figure 3.8.** A snapshot of a microgel (Sample C3) moving from a microchannel-at-large with a diameter of 180 μm to the constriction with a diameter of 50 μm. The microgel slows down and deforms at the entrance of the constriction. Scale bar is 100 μm.

### 3.3 Conclusion

We showed that a MF approach to the synthesis of IPN microgels with a narrow size distribution is a feasible approach to the preparation of microgels with IPN structures. In the present work, the microgels were prepared from the physically crosslinked alginate network and the chemically crosslinked PNIPAm. The effect of each variable in the microgels on the mechanical properties of the microgels was studied by varying its concentration in the feed solution. We found that the concentration of PNIPAm in the microgels and its degree of crosslinking did not have a significant effect on the mechanical properties of the microgels. However, alginate concentration in the microgels had a profound effect on their mechanical properties. Small increments in the concentration of alginate in the microgels yielded microgels with a wide range of Young’s moduli and relaxation times.
This work has implications in two fields. First, a method to measure the macroscopic mechanical properties in the microscopic scale was developed. The Young’s modulus and the relaxation time of the microgels were studied in AFM using a tipless cantilever, which prevented puncturing of the microgels. Second, the IPN microgels had a well-defined spherical shape, similar to some of the living cells and the Young's modulus values similar to living cells. In particular, the lowest Young’s modulus obtained in the present work was within the range of that for neutrophils. Therefore, these IPN microgels have the potential to be used as model system in studies of cells, as was demonstrated in their flow in microfluidic channels containing constrictions.
3.4 References


Chapter 4
Correlation between the Mechanical Properties of Microgels and their Flow in Confined Geometries

4.1 Introduction

The lungs microcirculation has a complex structure of capillaries branching out of the pulmonary arteries and converged to the pulmonary veins. The diameter of the pulmonary blood vessels decreases from 2.5-3.0 cm in the main pulmonary artery (pulmonary trunk) to 2 μm in the smallest capillaries.\textsuperscript{1,2} In addition to the difference in diameter of the different kinds blood vessels, their surface is also different. While the surface of arterioles (diameter of 10 to 125 μm) is coated with layers smooth muscle cells, the number of layers of these cells on the vessel walls decreases when they branch into the capillaries.\textsuperscript{3} In fact, there are no smooth muscle cells on the walls of capillaries, and the meta-arterioles, which are the connective vessels from the arterioles to capillaries, have only a discontinuous layer of smooth muscle cells. Instead, the walls of the capillaries are coated with a single layer of endothelial cells surrounded by collagen and mucopolysaccharides.\textsuperscript{3} The internal surface of blood vessels affects the mechanical properties of its walls. Commonly, the Young’s modulus of blood vessels walls is used to describe their stiffness. The Young’s modulus of smooth muscle cells was found to be 0.01-0.25 MPa whereas collagen was reported to be stiffer with Young’s modulus of 10-100 MPa.\textsuperscript{3}

Although the pulmonary microcirculation is unique and it differs from the systemic microcirculation in several aspects as was it described in Chapter 1, there are also similarities
between these microcirculations, such as the blood flow Reynolds number. The flow of blood in
the cardiovascular system is laminar with very low Reynolds numbers. The Reynolds number of
the flow of blood in the microcirculation varies with the diameter of the blood vessel. In an
arteriole with a diameter of 100 µm the Reynolds number is about 0.03 and it is lower in at least
one order of magnitude in the capillaries.1,3 The velocity of blood in the microcirculation was
reported as 5 mm/sec in the capillary sheet.1 Tested in cats, the velocity of blood in the arterioles
with diameter of 12 to 56 µm and capillaries with diameter of 8 to 10 µm was found to range
from 1.0 to 31.7 mm/sec and up to 4 mm/sec, respectively.1,3 Similar values were also measured
in rats with blood velocities of 0.8 to 12.9 and 0.2 to 1.2 mm/sec in the arterioles and capillaries,
respectively.1

The mechanical properties of neutrophils were discussed in details in Chapter 1. Briefly,
neutrophils are considered as relatively stiff blood cells. The Young's modulus of non-activated
neutrophils reported in the literature ranges from 200 to 2800 Pa.9,10 The elastic and loss shear
moduli of neutrophils measured in frequencies ranging from 0.1 to 102.4 Hz, were found to be in
the range from ~250 to ~708 Pa and ~80 to ~630, respectively.9 Under stimulation (either
chemical of physical stimulation) neutrophils may become stiffer or softer depending on the
nature of the stimulation.11 Also, the mechanical properties of the cells are affected by the
stiffness of the substrate used in the measurement.12 The viscosity of neutrophils reported in the
literature ranges from 6.5 to 89 Pa·s.13
In this chapter we describe a study of the flow of agarose microgels in microchannels containing a constriction as model system for the study of flow of neutrophils in lung capillaries. Specifically, this chapter describes the effect of the mechanical properties of the microgels on their flow through confined geometries. Monodisperse agarose microgels were prepared in microfluidic (MF) device by thermal gelation. The mechanical properties of the microgels were controlled by altering the concentration of agarose in the microgels, and tuned to be in the same range as neutrophils. The velocity of the microgels in the channel-at-large was also tuned to be similar to the velocity of blood in capillaries (with the diameter larger than 8 μm, excluding the diameter of neutrophils). The flow profile of each agarose microgels through constrained geometries was studied with respect to the microgel mechanical properties.

4.2 Results and Discussions

4.2.1 MF Synthesis of agarose microgels

Agarose microgel samples with different mechanical properties were prepared form solutions of 0.75, 1.0, 1.5 and 2.0 wt.% agarose in phosphate buffered saline (PBS, pH 7.4). The microgels were synthesized in a T-junction MF device\textsuperscript{4,5} by thermal gelation of agarose droplets.\textsuperscript{6} Droplets of agarose were generated in the T-junction by breaking up the stream of agarose solution by utilizing the shear imposed by the continuous phase of light mineral oil containing 3 wt.% of a nonionic surfactant Span-80. Droplets with diameter of 95 ± 5 μm were formed by adjusting the flow rate of the agarose stream to 0.09 mL/hr and the flow rate of the continuous phase to 0.8 – 1.2 mL/hr, depending on the concentration of agarose used. As was explained in Chapter 3, a higher flow rate ratio of the continuous-to-droplet-phase was used for droplet phase with higher
viscosity, i.e. higher concentration of agarose. Microgels were formed when the agarose in the droplets gelled upon cooling it from 37 °C in the syringe to 2 °C in the outlet tubing. The microgels dispersed in the light mineral oil were collected in a test tube containing a PBS buffer at 2 °C. The oil phase was removed after centrifugation of the sample at a rate of 1000 rpm for 15 min. The microgel samples used in the flow experiments were dispersed in PBS buffer.

4.2.2 Optical microscopy

An optical microscopy image of agarose microgels containing 1.0 wt.% agarose in PBS buffer is presented in Figure 4.1. The relative standard deviation in the microgels used in the flow studies was up to 4 %. The microgels obtained in the MF synthesis were spherical and translucent. Naturally, the optical contrast between the microgels and the PBS buffer medium increased with increasing the concentration of agarose in the microgels.

![Figure 4.1](image.png)

**Figure 4.1.** Optical microscope image of agarose microgels prepared from solution of 1.0 wt.% agarose and transferred to PBS buffer. Scale bar: 100 µm.

The flow of the microgels dispersions was studied in a microchannel containing a constriction. The details of the preparation of the microchannel for the flow experiments are given in *Chapter 2*. The rectangular cross-section microchannel with the height of 150 µm and the width of 162
and 52 μm in the channel-at-large and the constriction, respectively, was fabricated in poly(dimethylsiloxane) (PDMS) using a soft-lithography procedure. The cross-section of the channel was modified to produce a circular cross-section with a diameter of 150 μm in the channel-at-large and 50 μm in the constriction, respectively. Figure 4.2 shows a fluorescence microscopy image of the circular cross-section microchannel filled with an aqueous solution of 0.5 mg/mL fluorescent dye Rhodamine B. The modification of the cross-section of the microchannel did not alter the geometry of the microchannel both in the channel-at-large and in the constriction. However, the exit of the constriction of the modified channel did not have the same opening angle as the original microchannel, making the constriction asymmetric with respect to its entrance and exit angles. The entrance and exit angles to and from the constriction were 35 and 12 °, respectively.

**Figure 4.2.** A microchannel containing a constriction with a circular cross-section filled with a solution of fluorescent dye. The diameters of the channel-at-large and the constriction were 150 and 50 μm, respectively. The length of the constriction was 450 μm. The entrance and exit angle to and from the constriction were 35 and 12 °, respectively. Scale bar: 100 μm.

### 4.2.3 Mechanical properties of agarose microgels

The elastic shear moduli of macroscopic agarose hydrogels samples were measured using a rheometer with a plate-plate geometry (for detail see Chapter 2). Figure 4.3 shows the variation
in the elastic shear modulus, $G'$, of the hydrogels, which increased from 17.4 to 798 Pa when the concentration of agarose was increased from 0.75 to 2.0 wt.%. These values are within the range of elastic shear moduli of passive and stimulated neutrophils reported in the literature (the elastic shear modulus of passive neutrophils is about 250 Pa).\textsuperscript{9} We note that the elastic shear modulus measurements were performed in room temperature (T=25 °C) and that the moduli of the hydrogels in physiological temperature are smaller. However, in this current study, the flow of the microgels through microchannels containing a constriction was also studied in room temperature, and hence, the mechanical properties of the hydrogels were measured in the same temperature. The flow of the microgels through microchannels containing a constriction in physiological temperature will be studied in the future.

![Figure 4.3](image)

**Figure 4.3.** Variation in the elastic shear modulus of agarose hydrogels with the concentration of agarose.
4.2.4 Flow of agarose microgels in microchannels

The flow of each of the agarose microgels sample through a microchannel containing a constriction was studied with respect to their mechanical properties. The procedure of the flow experiments was described in details in Chapter 2. Briefly, the microgels were introduced in the microchannel with initial velocity of $2.60 \pm 0.15$ mm/sec, and the flow of the microgels was recorded using a high speed camera (at 300 frames per seconds). The experimental set-up was designed to mimic the flow of neutrophils in the microcirculation. The diameter of the microgels was smaller than the diameter of the channel-at-large (95 $\mu$m vs. 150 $\mu$m), to eliminate friction of the microgel with the channel walls in the channel-at-large. The ratio between the diameter of the microgel and the diameter of the constriction (95 $\mu$m vs. 50 $\mu$m) forced the microgels to deform to a similar extent as the deformation of neutrophils when they enter a small blood capillary. The microgels were injected in the microchannel using a syringe pump. The flow rate used in the experiments corresponded to the Reynolds numbers of $5 \cdot 10^{-4}$ and $1 \cdot 10^{-4}$ in the channel-at-large and the constriction, respectively. These Reynolds numbers are comparable with the Reynolds numbers in the microcirculation.\textsuperscript{1,3}

A snapshoot of a microgel containing 1.5 wt.% agarose, flowing through the channel-at-large toward the constriction is shown in Figure 4.4. The flow profiles of 0.75, 1.0, 1.5 and 2.0 wt.% agarose microgels are shown in Figure 4.5. The velocities of the microgels in the profiles were normalized with respect to their initial velocity in the channel-at-large (before the constriction). We distinct 6 regions in the flow profiles of the microgels in the microchannel. Generally, all the microgel samples were flowing through the channel-at-large before the constriction at a constant velocity, this part of the channel is marked on the flow profiles as Zone 1. As the microgels
reached the tapered part of the microchannel toward the entrance of the constriction (Zone 2), they accelerated due to the increase in the velocity of the liquid. In Zone 3, at the entrance of the constriction, the microgels either stopped or slowed down, depending on their stiffness, in order to deform to the lower diameter of the orifice. Then the microgels flowed through the constriction (Zone 4) with a velocity that was depended on their stiffness. In Zone 5, at the exit of the constriction, where the diameter of the microchannel was increased back to the diameter of the channel-at-large, all four samples showed an increase in the velocity. This behavior was caused by the higher velocity of the liquid and the gradually disappearance of the physical constrain imposed by the constriction. Finally, in Zone 6 the velocity of the microgels decreased back to, or close to the value of their initial velocity at the channel-at-large before the constriction.

Figure 4.4. A snapshot of 1.5 wt.% agarose microgel flowing in the channel-at-large toward the constriction. Scale bar: 100 µm.
Figure 4.5. Flow profiles of agarose microgels flowing through a microchannel containing a constriction. The profiles show the microgels velocity normalized to their initial velocity at the channel-at-large before the constriction against the distance of the microgels from the center of the constriction. a) 0.75 wt.% agarose microgels (G’=17.4 Pa), b) 1.0 wt.% agarose microgels (G’=92 Pa), c) 1.5 wt.% agarose microgels (G’=362 Pa) and d) 2.0 wt.% agarose microgels (G’=798 Pa). Red lines show a line connecting the data points on the flow profiles; black dotted lines connect points of error calculated by the standard deviation of the mean.

As can be seen in Figure 4.5 and Figure 4.6 that compares the flow profiles of the four microgel samples, there are substantial differences between the flow patterns of the microgels with
different mechanical properties through the microchannel. Although all samples flow toward the constriction with similar initial velocity and all show an increase in the normalized velocity in the tapered part of the microchannel before the constriction, the extent of increase in the normalized velocity in Zone 2 depended on the mechanical properties of the microgels. As the concentration of agarose in the microgels increased (i.e. the value of G’ increased) the increase in the normalized velocity in Zone 2 of the microchannel was smaller. This effect was caused by the difference in the relaxation time of the microgels, which, increases with increasing G’. As the relaxation time of the microgels increased, they required longer times to respond to the physical constrain due to the decrease in the diameter of the microchannel. Hence, the velocity of the microgels with the longer relaxation time did not increase together with the velocity of the liquid as rapidly as the microgels with the sorter relaxation time. It should be noted that this effect is clearly seen in the flow of the stiffest and softest microgels (G’=17.4 Pa and G’=798 Pa, respectively), whereas, the normalized velocities of the microgels samples with values of G’=92 Pa and G’=362 Pa were too similar to each other.
Figure 4.6. Superimposed flow profiles of all agarose microgel flowing through a microchannel containing a constriction. a) Full flow profile. b) An inset of the constriction entrance. The noise in the results in the inset graph was reduced using the denoise function in Origin. —— 0.75 wt.% agarose microgels ($G'=17.4$ Pa), —— 1.0 wt.% agarose microgels ($G'=92$ Pa), —— 1.5 wt.% agarose microgels ($G'=362$ Pa) and —— 2.0 wt.% agarose microgels ($G'=798$ Pa).
The second effect of the mechanical properties on the flow pattern of the microgel through the microchannel is seen in the reduction of the normalized velocity at the entrance of the constriction (Zone 3, Figures 4.5 and 4.6 and Table 4.1). In order to enter into the constriction that had a diameter of 50 μm, the microgels that had a diameter of 95 ± 5 μm, must deform to about 50% of their original diameter. Interestingly, although the elastic shear modulus of the microgels was increased less than 50 times from the softest to the stiffest sample, the behavior of the microgels in Zone 3 was markedly different. We find these results surprising since we expected all microgel samples to stop at the entrance of the constriction. However, the flow profiles show zero velocity only for the stiffest sample. A slight reduction in the normalized velocity (in about 30% of the initial velocity) was observed for the two intermediate samples and a very small reduction in the normalized velocity was seen for the softest microgels. In the case of the softest microgels, the reduction in the velocity was so small, that the velocity did not even go below the initial velocity (Table 4.1). As the microgels become stiffer, they require longer time for the deformation at the entrance of the constriction, thus their normalized velocity decreases more and even goes down to zero for the stiffest sample.

Table 4.1. Elastic shear modulus, normalized velocity and stall time at the entrance of the constriction (Zone 3) of agarose microgels.

<table>
<thead>
<tr>
<th>C_{agarose} (wt.%)</th>
<th>G’ (Pa)</th>
<th>Normalized velocity at the entrance of the constriction (Zone 3)</th>
<th>Stall time at the entrance of the constriction (Zone 3) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>17.4</td>
<td>1.27 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>92</td>
<td>0.68 ± 0.23</td>
<td>0</td>
</tr>
<tr>
<td>1.5</td>
<td>362</td>
<td>0.77 ± 0.05</td>
<td>0</td>
</tr>
<tr>
<td>2.0</td>
<td>798</td>
<td>0</td>
<td>7.1 ± 5.9</td>
</tr>
</tbody>
</table>
The third and the clearest difference in the flow behavior between the four microgel samples was seen during the flow through the orifice (Zone 4), as can be seen in Figures 4.5 and 4.6. As the concentration of agarose in the microgels increases, the normalized velocity of the microgels in the orifice becomes smaller. Since the friction coefficient of the microgels increases with increasing the concentration of agarose in the microgels, the counter force to the flow on the softer microgels in the constriction was smaller. Thus, these microgels flowed through the constriction at higher normalized velocities than the stiffer microgels.

The last effect of the mechanical properties on the flow profiles of the microgels is seen in Zone 5 at the exit from the constriction, where the microchannel is widen form a diameter of 50 μm back to 150 μm, which is the diameter of the channel-at-large. As seen in Figures 4.5 and 4.6, as the concentration of agarose in the microgels increases, the normalized velocity at the exit of the constriction decreases. This effect is also attributed to the difference in the relaxation time between the microgels, similar to the effect seen in the flow behavior at the entrance of the constriction. We note that as in Zones 2 and 3, this effect shows three populations (softest, intermediate and stiffest which correspond to fast medium and slow normalized velocities) rather than four populations. Also, the increase in the normalized velocity at the exit of the constriction is larger than its increase at the entrance of the constriction. That might be an effect of the opening angle due to the asymmetric structure of the constriction. Since the exit angle from the constriction is smaller than the entrance angle (12° vs. 35°) the reduction of the velocity of the liquid at the exit of the constriction is slower, giving the microgels longer time to response to the removal of the physical constrain imposed by the microchannel walls.
Finally, in Zone 6, the diameter of the microchannel goes back to its initial value and all microgels slow down. Although expected, the velocity of the 0.75 wt.% agarose microgels does not go back to its initial value. That might be due to the limited view of microchannel under the microscope and that the microgels slow down to their initial velocity while they flow downstream in the microchannel.

4.3 Conclusions

This chapter describes the effect of mechanical properties of agarose microgels on their flow through a microchannel containing a constriction. The elastic shear modulus of the microgels were tuned be in the same range as neutrophils by altering the concentration of agarose in the microgels. The microgels were synthesized in a T-junction MF device and their sizes were tuned to be 95 ± 5 μm, in order to force them to deform at the entrance of the constriction in the microchannel to the same extent neutrophils deform while they enter a small blood capillary. In addition, the initial velocity of the microgels in the channel-at-large before the constriction was tuned to be in the same range as the velocity of blood in the microcirculation.

The flow profiles of the microgels through the microchannel were studied in light of their mechanical properties. It was found that the stiffness of the microgels determined the normalized velocities in the constriction. The effect of relaxation time of the microgels on their flow through the microchannel was observed in the increase in the velocity of the microgels in the tapered regions before and after the constriction. However, in these cases, the two intermediate samples showed similar velocities.
One important point that should be taken into account in future modeling is the difference between the mechanical properties of blood vessels and the model microchannel. Although the mechanical properties of the microgels were tuned to the same range of neutrophils, the mechanical properties of the microchannel walls were not similar to the mechanical properties of blood capillary walls. In fact, the elastic modulus of PDMS is about an order of magnitude higher than the modulus of smooth muscle cells and one to two order of magnitudes lower than the modulus of collagen.\textsuperscript{17,18}
4.4 References


Chapter 5
Conclusions and Future Work

5.1 Conclusions

This thesis describes the synthesis of two different types of microgels in microfluidic (MF) approach. Alginate-poly(N-isopropylacrylamide) (PNIPAm) interpenetrating polymer network (IPN) microgels were synthesized in a flow-focusing MF device; agarose microgels were synthesized in a T-junction MF device. MF enables synthesis of particles with control over their size, shape and morphology. In both cases, precursor droplets were formed “on chip” and then gelled into microgels while flowing downstream in the MF device. The IPN microgels formed when Ca\(^{2+}\) and tetramethylethylenediamine diffused into the droplets and cross-linked alginate and polymerized NIPAm. In the case of agarose microgels, the agarose gelled while the droplets were cooled down from 37 °C to 2 °C. The microgels described here were prepared in series of samples with varied mechanical properties. The mechanical properties were controlled by altering the concentrations of the components in the microgels.

The mechanical properties of the IPN microgels were studied using atomic force microscopy (AFM). A Method to measure the Young’s modulus and relaxation time of individual microgels were developed. The mechanical properties were measured using a tipless cantilever and were done under fluid. The significance of this method is its ability to measure macroscopic properties
of an individual microgel in the microscopic scale. The Young’s modulus of the IPN microgels was tuned to be in the same range as living cells in general, and neutrophils in particular.

The mechanical properties of agarose hydrogels were measured using a plate-plate configuration rheometer and was performed on macroscopic samples. The elastic shear modulus of the agarose hydrogels was tuned to be in the same range as passive and activated neutrophils.

The application of IPN microgels as artificial cells in cells studies was demonstrated by their flow through a microchannel containing a constriction. The flow of agarose microgels under constrained geometries was studied in greater details. All microgels showed an ability to deform to smaller diameter in order to enter a constriction 50% smaller than their initial diameter. Agarose microgels showed correlation between their mechanical properties and the flow in the microchannel. As the microgels became softer they showed a smaller reduction in their velocity at the entrance of the orifice. Similarly, the softer microgels flowed through the orifice at higher velocities. The relaxation time was also found to affect the flow of agarose microgels. As the concentration of agarose in the microgels increased, i.e. the relaxation time increased, the velocities of the microgels at the tapered parts of the microchannel before and after the constriction decreased.
5.2 Future work

The study of flow of hydrogels under constrained geometries as model system in cells studies is in its preliminary stage. This study has plenty of directions to evolve to. In parallel with the study of effect of mechanical properties, other effects are currently being investigated. The effect of microgel initial velocity, constriction opening angle and microgel size are being studied. In this approach, of studying each variable in the system separately, we can assess the contribution of each effect to the passive mechanism of neutrophils deformation. In the nearest future, modeling of the current results should be done. The results should be fitted to equations describing the role of mechanical properties in the flow of the microgels through the microchannel. Also, a measurement of the relaxation time of agarose hydrogels will contribute to a complete understanding of the flow profiles of the hydrogels.

In later stages, the design of the model system of the microchannel should be designed to better resemble the real system with its complexities. The microchannel should have multiple constrictions and the flow of microgels through microchannel with branching constrictions should be studied. Secondly, the validity of scaling up the microgels and microchannels size should be confirmed by scaling down the dimensions of the model system. Lastly, the weight of passive mechanisms on the deformation of neutrophils can be assessed by performing similar experiments with real neutrophils and comparing the flow profiles of microgels that only passively deform and cells that deform simultaneously in active and passive manners.