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Abstract
During the course of a PhD, many research ideas are pursued that do not become successful or completed projects. Even though some of these are not developed enough to become chapters in this thesis, they are still promising directions for future research. In this final chapter, I outline a couple of those projects.


9.1 Air-filled microspheres

In this thesis, we have used AFS to apply forces on polystyrene microspheres. Polystyrene microspheres are advantageous because of their commercially availability in a wide range of sizes, their mono-disperse size distribution and the possibility to couple them to various chemical labels. As shown in Figure 2.2a, different materials have different acoustic properties and polystyrene does not have the strongest response to acoustic fields. While we have optimized our chip configuration in Chapter 3, we have never optimized the microsphere material in order to get the highest efficiencies. A higher efficiency in force with the same size microsphere means, of course, that higher forces can be reached. Another possibility is to reduce microsphere size, which results in a faster response time and a higher localization accuracy of the system (section 2.4.5 and 2.4.6).

To quantify how strong material responds to the acoustic field, the acoustic contrast factor ($\Phi$) is used:

$$
\Phi = \frac{\rho_p}{2 \rho_p + \rho_m} + \frac{1}{3} \frac{\rho_m c_m^2}{\rho_p c_p^2}
$$

(9.1)

With $\rho_p$ and $\rho_m$ being the densities, and $c_p$ and $c_m$ being the speed of sound of the particle and the medium, respectively. A polystyrene particle has an acoustic contrast factor of 0.22, denser and stiffer material like glass 0.54 (Table 9.1). Since the acoustic radiance force scales with the volume of the object (equation 3.2), the decrease in particle size for a glass microsphere that can be used with AFS is limited.

Inspired by the field of ultrasound imaging, we have been testing acoustic contrast agents. These are air-filled particles, often made from lipids, with a very high acoustic contrast factor, due to the high difference in density of air compared to water (Table 9.1). Note that the contrast factor is negative, meaning that these particles are pushed to the anti-node of the acoustic field. However, the compressibility of air is also very high, as a result, these particles generate a strong acoustic field around them that interacts with other particle or the surface, called secondary Bjerknes forces. These secondary effects could interfere with our measurement. For this reason, we have set out a search to find air-filled particle with a low compressibility. Dmitry Grishenkov (Department of Medical engineering, the Royal Institute of Technology, Sweden) has provided us with air-filled polymer-shelled microspheres. These microspheres have an average size of 2 $\mu$m in
Figure 9.1 Calibration of acoustic forces acting on air-filled microspheres
(a) Graph showing a cross section of the flow cell. The acoustic pressure profile (red line) is plotted of the 10.3 MHz resonance frequency, using the bottom force chip described in Chapter 3. Silica and air-filled microspheres are pushed toward the node and the anti-node, respectively. (b) Graphs showing the height location of the silica (bottom) and air-filled (top) microspheres over time, while applying different voltages. (c–d) Measured (dots) and fitted (line) force profile for the silica (c) and the air-filled (d) microspheres plotted for three different voltages. (e) Distribution of the calibrated force/voltage² relation of the air-filled and the silica beads. Distributions are fitted with a Gaussian function, yielding a peak at 0.94 ± 0.01 and 1.7 ± 0.2 pN V⁻², and a width of 0.69 ± 0.03 and 1.8 ± 0.5 pN V⁻² for the silica and air-filled microspheres, respectively. (f) Calibrated force/voltage² relation plotted versus the measured microspheres size and fitted with a third power function. Fit yields 4.3 ± 0.2 pN V⁻² μm⁻¹. (fit values ± s.e.m. for c–f)
diameter, the shell is approximately 300 nm thick, made of poly-vinyl alcohol (PVA) and the reported acoustic contrast factor is \(-60.7\). To test if these particles are suitable for AFS experiments, we calibrate their response in the AFS setup.

We observe that these air-filled microspheres are floating upwards to the top of the fluidic chamber, in contrast to other microsphere that are heavier than water and sink to the bottom. To calibrate the force applied to these microspheres, we used the method explained in section 3.4.4, where the microspheres are forced from the surface to the acoustic node. To calculated the acoustic contrast factor, we compare the acoustic response of the air-filled microspheres to microspheres with a well-known acoustic contrast factor (silica microspheres with 6.8 μm in diameter). To make a correct comparison, we calibrate both microspheres with the same resonance frequency in the same chip. To this end, we use a resonance frequency that pushes the air-filled microspheres downwards to the acoustic anti-node and the silica microspheres upwards to the acoustic node (Figure 9.1a). We observe that the air-filled and the silica microspheres can be forced in a controlled fashion to the acoustic anti-node and node, respectively (Figure 9.1b). The force on the microspheres at each height location is determined from the velocity with which they move from the surface to the node (or anti-node), as explained in section 3.4.4. The force profiles for different applied voltages are fitted with sine functions (Figure 9.1c and d) and the force/voltage\(^2\) ratio is calculated for a population of silica and air-filled microspheres (Figure 9.1e).

We notice a large spread in the force/voltage\(^2\) ratio for the air-filled microspheres. Therefore, we use the upward velocity of the air-filled microspheres to calibrate each individual radius. To this end, we set out a force balance of all the forces experienced by the microsphere: the buoyance \((F_b)\), gravitation \((F_g)\) and the stokes drag force \((F_{\text{Stokes}})\) and solved it for the velocity:

\[
F_b + F_g + F_{\text{Stokes}} = 0 \quad (9.2)
\]

\[
F_g = V\rho g = \frac{4}{3}\pi g \left( (R_2^3 - R_1^3)\rho_{\text{PVA}} + R_1^3\rho_{\text{air}} \right) \quad (9.3)
\]

\[
F_{\text{Stokes}} = -6\pi\eta R_2 v \quad (9.4)
\]
\[ v = \frac{2g}{9\eta R_2} \left( (R_2^3 - (R_2 - d)^3)\rho_{PV4} + (R_2 - d)^3\rho_{air} - R_2^3\rho_{water} \right) \quad (9.5) \]

Where \( V \) represents the volume of the microsphere, \( \rho \) the density, \( R_1 \) and \( R_2 \) the inner and the outer radius of the microsphere, respectively, \( \eta \) the viscosity of the medium and \( d \) the shell thickness \((R_2 - R_1 = 300 \text{ nm})\). Using equation 9.5, the outer shell radius could be extracted from the floating velocity. Since the acoustic force scales with the volume of the particle, the force/voltage\(^2\) ratio is plotted against the inner radius and fitted with a third power function (Figure 9.1f). When we extrapolate this function to the radius of the silica microspheres \( (3.4 \mu m) \), we find that the air-filled microspheres experience \( 170 \pm 14 \) fold higher force than the silica ones, but in the opposite direction. As a result, we find that the acoustic contrast factor is \(-170 \pm 14 \cdot 0.54 = -92 \pm 7\) compared to polystyrene microspheres (the microspheres material we typically use). The increase in force is about 400-fold, which means that we can use at least 7 times smaller microspheres and still reach the same force.

If those air-filled microspheres could be used in combination with AFS, it would provide an enhancement in the response time of the system and localization accuracy (see section 2.4.5 and 2.4.6). However, they are at the moment not commercially available. Furthermore, we have not managed yet to chemically couple them to a biological sample and, lastly, the AFS chip is optimized to apply forces on particles with a positive acoustic contrast factor. These issues have to be overcome in order to make the air-filled microspheres suitable for experiments.

<table>
<thead>
<tr>
<th></th>
<th>Density ((\text{Kg m}^{-3}))</th>
<th>Speed of sound ((\text{m s}^{-1}))</th>
<th>Acoustic contrast factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1000</td>
<td>1482</td>
<td>-</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>1050</td>
<td>2350</td>
<td>0.22</td>
</tr>
<tr>
<td>Glass</td>
<td>2230</td>
<td>5674</td>
<td>0.54</td>
</tr>
<tr>
<td>Air</td>
<td>1</td>
<td>332</td>
<td>-6662.</td>
</tr>
<tr>
<td>Cell</td>
<td>1100</td>
<td>1500</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 9.1 | Acoustic contrast factor \((\Phi)\) for different materials.
Acoustic contrast factor calculated using equation 9.1. Values for the density and speeds of sound are taken from Mikkel Settnes and Henrik Bruus\(^{18}\). Note, that there many different kind of cells and that the acoustic contrast factor can vary between cell types\(^{15}\).

Another potential application of these microspheres could be to chemically modify them in such a way that they bind to specific parts inside a cell. When an acoustic field is applied, these microspheres generate force inside a living
cell that can be controlled with the applied acoustic field. Because of their ability to generate large forces, even when the particles are small, forces can be applied at specific locations in the cell.

Acknowledgements
We thank Dmitry Grishenkov (Department of Medical engineering, the Royal Institute of Technology, Sweden) for providing the air-filled microspheres.

9.2 Mechanical measurement on Red Blood Cells (RBCs)

This section (9.2) is based on: Raya Sorkin*, Giulia Bergamaschi*, Douwe Kamsma, Guy Brand, Elya Dekel, Yifat Ofir-Birin, Neta, Regev-Rudzki, Wouter Roos and Gijs J.L Wuite; Probing cellular mechanics with Acoustic Force Spectroscopy; manuscript submitted in Molecular Biology of the Cell

Mechanical properties of cells are essential for their function and response to the environment. Differences in stiffness can be related to several diseases, such as cancer, anemia or malaria. However, current methods to study mechanical properties of cells, like atomic force spectroscopy, fluid flow experiments or optical tweezers, lack data throughput making it a tedious process to distinguish mechanical properties in a heterogeneous population.

Preliminary data shows that AFS can be used to study the mechanical properties of many Red Blood Cells (RBCs) in parallel. The RBCs are attached to the bottom of the flow chamber functionalized with Poly-L-lysine. Concanavalin A functionalized silica microspheres (6.8 μm in diameter) are placed on top of the cells (Figure 9.2a-b). When the acoustic force is applied, the microspheres are forced upwards and thereby pulling on the membrane of the RBCs (Figure 9.2c). We use relatively large silica microsphere to have a high imaging contrast compared to the RBCs (Figure 9.2d) for 3-dimensional tracking and to have a higher acoustic contrast factor than RBCs (Table 9.1). The microspheres are tracked in 3 dimensions using the same tracking software as described in Chapter 4. In a typical experiment, we apply a constant load to the RBCs and track their elongation over time (Figure 9.2e). The response of the RBCs to load matches with data obtained on lipid vesicles with fluid flow experiments. In these experiments, membrane tethers are pulled out of lipid vesicles and since we also observe such a high elongation. Since we observe elongation rates similar to those observed on lipid vesicles
Figure 9.2 | Measuring mechanical properties of surface-attached RBC using silica microspheres
(a) The AFS chip is imaged using an inverted microscope with objective lens (OL), a digital CMOS camera and LED light source (625 nm). (b) Side view of the bottom part of flow cell. The flow cell is coated with Poly-L-lysine, RBC are attached to the functionalized surface. Silica microspheres, 6.8 μm in diameter, are functionalized and placed on top of the RBC. (c) The acoustic force is applied and the microspheres are pushed up toward the acoustic node, resulting tether formation on the RBC. (d) Digital camera images of the field of view (1) and zoom-in to a region with four tracked microspheres (black squares) (2). (e) Example curve of the elongation of the RBC (black dots), while applying the acoustic force. The elongation is fitted with equation 9.6. (red line). (f) Visualisation of the Burger’s model described with dashpots and springs. (g) Chemical structure of formaldehyde (left) and 7KC (right) and the effect on spectrin and lipid bilayer. (h–j) Distributions of the $L_0$ (h), $L_{cross}$ (i) and $τ_{rel}$ (j) for healthy RBC, incubated with 7KC (5 μM) and formaldehyde (0.04% w/v). Only $L_0$ show significant differences.
Therefore, we use Burger’s viscoelastic model\textsuperscript{13} to quantify the response of the RBCs:

\[
L(t) = L_0 + L_{cross} \cdot \left[ 1 - \exp \left( -\frac{t - t_0}{\tau_{rel}} \right) \right] + L'_v(t - t_0)
\] (9.6)

Where \(L_0\) is the instantaneous response to the load, \(L_{cross}\) the retarded elastic behaviour, \(\tau_{rel}\) the response time of the retarded elastic behaviour and \(L'_v\) the long term viscous flow (Figure 9.2f).

As a proof of principle, we tested the capabilities of this method by detecting changes in cellular mechanics of RBCs treated with formaldehyde and 7-ketocholesterol (7KC) (Figure 9.2g). Formaldehyde crosslinks the spectrin network of the RBCs causing it to stiffen\textsuperscript{14} and 7KC penetrates into the lipid bilayer making it less viscous\textsuperscript{14}. From our experiments, we observe that the \(L_0\) significantly increases by the treatment with formaldehyde or decreases with 7KC, while the other parameters are not affected (Figure 9.2h-j). For formaldehyde, this result is expected, because a stiffer network will reduce the elastic elongation response of the RBCs. For 7KC, we expected that \(L'_v\) changes, because a less viscous lipid bilayer would be easier to stretch. Possibly, the less viscous lipid bilayer is more wobbly around the cell, explaining the higher instantaneous response.

These results show that AFS can be used to study the mechanical properties of RBCs in a multiplexed fashion, paving the way to new insights into cell mechanics and mechanobiology. The method can easily be extended to other systems, broadening the spectrum of possible applications of AFS.

\textbf{Acknowledgements}

The experiments in this section (section 9.2) were performed together with Raya Sorkin and Giulia Bergamaschi (and will be continued by them).

\textbf{9.3 Measuring the adhesion strength and migration of cells at 37°C}

In Chapter 7 we have demonstrated that AFS can be used to directly apply forces to cells and thereby measure their adhesion strength and migration. We called this new approach single-cell Acoustic force spectroscopy (scAFS). The measurements in Chapter 7 were performed at room temperature (23°C), making the results obtained less directly biologically relevant. In this section,
we show that it is straightforward to perform scAFS measurements at 37°C.

To this end, we use a temperature controlled flow cell holder (see section 6.2.3) to bring the sample chamber to 37°C and we adjust the resonance frequency of the chips accordingly (Supplementary figure 6.3). The sample chamber is modified with fibronectin to mimic the ECM (described in section 7.4.2). In collaboration with Hanke Matlung and Michel van Houdt (Sanquin, Netherlands), we measured the cell adhesion strength and migration of a promyelocytic cell line (NB4) that was differentiated towards neutrophil-like cells by stimulating them for 7 days with all-trans retinoic acid. Data was obtained in one day of experimentation, as described in Chapter 7. Data on adhesion strength, spatial movement and velocities of the cells are shown in Figure 9.3.

We measured on five different NB4 cell populations: wild-type (WT) cells, Phorbol Myristate Acetate15 (PMA) activated wild-type cells, CD18 Knock Out cells (CD18KO; prepared via CRISPR and Cas9), CD18KO cells reconstituted with WT CD18 (via lentiviral transduction) or CD18KO cells reconstituted with a mutated form of CD18, containing mutations in the cytoplasmic NxxY/F motif, resulting in a loss of binding of kindlin-3 and talin16. In general, we observe clear correlation between adhesion strength (Figure 9.3a) and a cell's ability to move as well as its velocity (Figure 9.3bc). WT NB4 cells function as baseline, and we clearly observe that the PMA activation, which activates CD18 integrins, increases their binding strength, while the total movement and cell velocity decreases. For the CD18KO we observe a larger spread in all parameters obtained, however, the median values remain the same. Interestingly, the reconstituted cells show a reduced adhesion strength, probably as a result of less integrin-mediated adhesion points, possibly due to incomplete reconstitution. Finally, the mutated cells show less adhesion strength compared to the wild type. This result is expected, because the mutated integrin is unable to bind kindlin and talin, which are needed for integrin mediated adhesion16. Even though a lot of useful data is generated in one day of experimentation, further analysis, interpretation and comparison with established methods are needed to validate and understand these results.

This study shows that adhesion strength and migration of single cells can be measured with scAFS at 37°C in a fast and straightforward fashion. As already discussed in Chapter 7, scAFS has a great potential in the field of cell adhesion. Showing that these measurements can be performed on 37°C is a small, yet
Figure 9.3 | Cell-adhesion strength and migration measured at 37°C.
5 different NB4 cell populations are measured: wild type cells (turquoise), PMA activated wild type cells (purple), b2 integrin knockout (CD18KO) (red), reconstituted cells (blue) and mutated cells that cannot bind kindlin-3 and talin (green). (a) Boxplot of the measured adhesion strength. Data is obtained as described in chapter 7. Whiskers are maximum and minimum, and quartiles framing the upper and lower part of the median. (b) Histograms of the average movement of the cells before applying a strong force ramp. Standard deviation of the cell position is calculated using a rolling window that continuously samples 225 data points and averaged for each cell (data acquisition rate is 60 Hz). (c) Histograms of the average velocity of the cells before applying a strong force ramp. The in-plane velocity is calculated using rolling window of 225 data points and is averaged for each cell.

very important step with respect to the physiological relevance of the data: it substantially expands the possible application of scAFS in research purposes or even medical diagnostics.

Acknowledgements
The work here (section 9.3) is performed in collaboration with Raya Sorkin (VU), Hanke Matlung (Sanquin, Netherlands) and Michel van Houdt (Sanquin, Netherlands).
Measuring the adhesion strength and migration of cells at 37°C.