

1. Results

1.1. Virtual Prototyping of Cell Signals

During the course of this thesis, numerical simulations for the microchannel have been carried out in MATLAB. First, a simulation about the shape of a giant magneto resistance (GMR)-sensor signal of cells was performed, where the magnetic moment was conveyed through magnetic nanoparticles (MNPs) bound to their surface. Second, cell aggregates have been looked at in the same manner with different angles respective to the sensor. Third, both simulations were correlated to a reference dipole, with the equivalent magnetic momentum located in the center of mass.

Additionally, the flow and shear field inside the channel was simulated numerically for the channel cross-section as well as for a particle near the walls. A force equilibrium simulation was also established in a basic manner.

All simulations have been captured inside the MATLAB class “MRCyte”, which contains material parameters, constants and the necessary functions for all simulations above.

1.1.1. Numerical investigation of immunomagnetic label density and size on quantitative magnetoresistive sensing of single cells and cell aggregates

In order to mimic a immunomagnetically labeled cell flowing over the sensor half bridge, the planar integral of the respective magnetic flux density (\mathbf{B}) was solved analytically. Here, \mathbf{r}_i specifies the distance vector of a single MNP from the sensor plane. The magnetic flux density was converted to a resistive change \mathbf{R}_{sig} by scaling it with the GMR-sensitivity S and subsequently into a signal voltage \mathbf{V}_{sig} inside the bridge branch.(Eqs. 1.1 to 1.3)

First, MNPs were randomly sampled on a sphere surface with an diameter of $4 \mu\text{m}$ or $8 \mu\text{m}$. Then, the signal was computed from the superposition of every MNP during each timestep. Additionally, the MNP distribution was rotated in every iteration to resemble a rolling motion. The computed signals were then cross-correlated to the signal of a reference flux density \mathbf{B}_{ref} caused by a point-like magnetic momentum located in the geometric center of the same sphere.

$$\mathbf{B}(t) = \sum_{i=1}^N \frac{1}{A_{\text{Sensor}}} \int_{-\frac{l}{2}}^{\frac{l}{2}} \int_{-\frac{w}{2}}^{\frac{w}{2}} \frac{\mu_0}{4\pi} \left(\frac{3\mathbf{r}_i(t) (\mathbf{r}_i(t) \cdot \mathbf{m}_i)}{|\mathbf{r}_i(t)|^5} - \frac{\mathbf{m}_i}{|\mathbf{r}_i(t)|^3} \right) dx dy \quad 1.1$$

$$\mathbf{R}_{\text{sig}}(t) = -\mathbf{B}(t) \times \frac{S}{100} \times R + R \quad 1.2$$

$$\mathbf{V}_{\text{sig}}(t) = \frac{\mathbf{R}_{\text{sig}}(t)}{R + \mathbf{R}_{\text{sig}}(t)} \times V_p - \frac{V_p}{2} \quad 1.3$$

By its formula, cross-correlation $R_{xy}(\tau)$ yields a displacement dependent signal through its convolution of the complex conjugated reference signal $V_{ref}^*(t)$ with the sample signal $\mathbf{V}_{sig}(t + \tau)$. (Eq. 1.4) Therefor, only the maximal correlation of this function was considered in further analyses.

$$\max\{R_{xy}(\tau)\} = \max \left\{ \int V_{ref}^*(t) \mathbf{V}_{sig}(t + \tau) dt \right\} \quad 1.4$$

1.1.2. Single Cell Signal

Aim of these simulations is to find a measure of how magnetic labeling of a cell affects signal shape and its subsequent analysis. A single cell with a surface coverage of 5 % to 99 % of a densely packed sphere was loaded randomly with MNPs at different sizes. Then, the previously explained rolling motion over the sensor bridge was simulated with the parameters specified in Table 1. After correlating of the resulting signal voltage to the reference dipole (Fig. 1b, ●), each with three equal, randomly distributed MNP coverages, the dependency on the coat was evaluated. As shown in the schematic Fig. 1b, an increase in signal peak amplitude but also in full width at half maximum (FWHM) at growing coverage was expected .

The expected behavior matches the data analysis (Fig. 2). Each two analyzed sphere diameters 4 μm and 8 μm with MNP sizes ranging from 20 nm to 2 μm , show a great standard error of the mean (SEM) at low coverage. This very probably is subjected to

Parameter	Unit	Value	Explanation
w	m	2.0×10^{-6}	GMR width
l	m	30.0×10^{-6}	GMR length
d	m	14.0×10^{-6}	Distance between two sensors
R	Ω	250	GMR Resistance
V_p	mV	100	Supply voltage
$t_{free layer}$	m	7.0×10^{-9}	Thickness of free layer
M	A m^{-1}	2.0×10^4	Volume Magnetization
$V_{noise,rms}$	V	2.5×10^{-6}	Artifical noise
Sim. Space	m	$[-25 \times 10^{-6}, 25 \times 10^{-6}]$	Interval around sensor center

Table 1: Magnetic Simulation Parameters

Constants used inside the framework for the simulation of the magnetic field inside the GMR Wheatstone half bridge. The volume magnetization was adapted according to the simulated particle size.

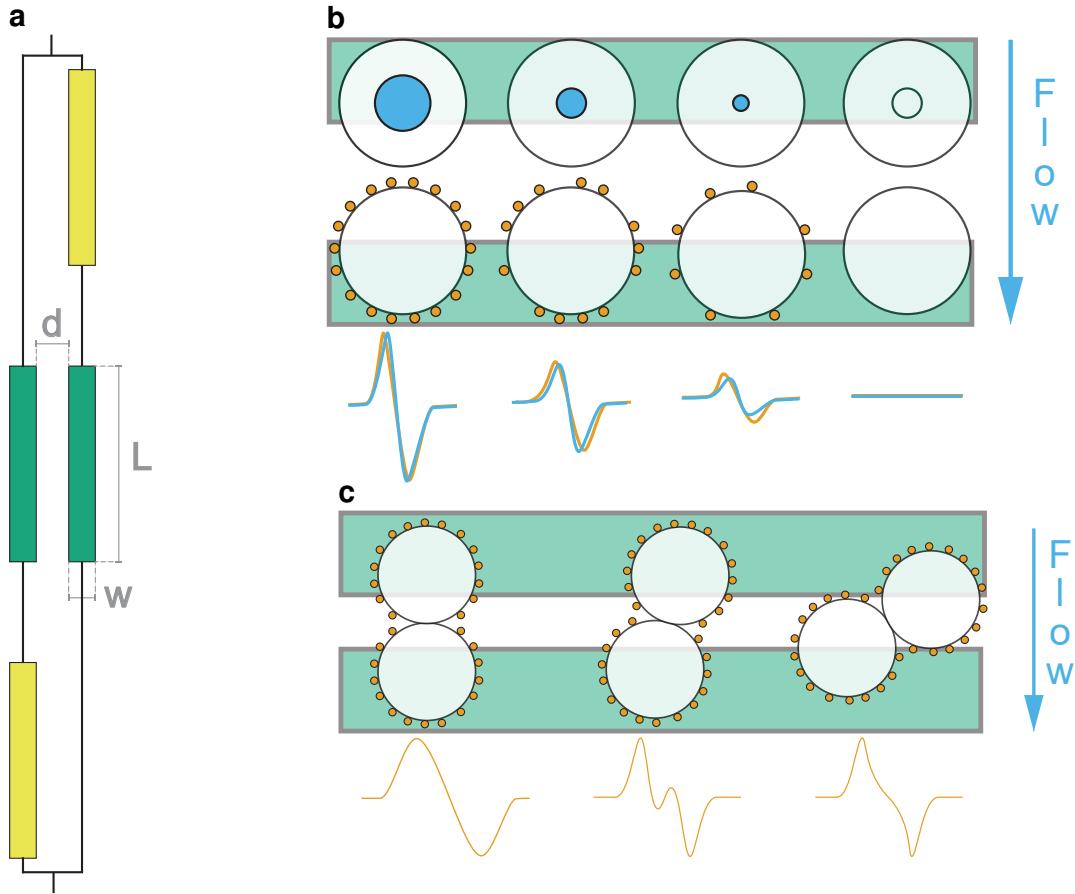


Figure 1: Particle Coverage Simulation

(a) Dimensions of the GMR Wheatstone bridge sensor: Distance d between both variable bridges (green), width w of a GMR-sensor, length L of a sensor. (b) Scheme of single cell simulation: The ideal magnetic dipole in the geometric center of a sphere (●) causes a signal deviation from the real cell signal with magnetic moment distributed on the cell surface. (●) (c) Signal shapes of different angles of two-particle aggregates lead to differing signal shapes.

the momenta of single particles which play a greater individual role and hence influence the signal shape significantly because the overall dipole momentum in the sensor loses homogeneity.

Another observable effect is related to the MNP size. Absolute correlation differs from 20 nm to the ten and hundred fold diameter significantly. This can be related to the magnetic momentum per MNP as it is dependent on the volume - thus r^3 . However, for bigger magnetic particles this does not completely hold true because the composition changes from pure magnetite to a polymer shell with embedded oxide core at around 150 nm. Nevertheless, larger particles carry also greater magnetic momentum which brings the aforementioned influence of single MNPs into consideration.

Further, the densely packed sphere surface can evidently carry more smaller than larger MNPs. This ranges from 641 600 MNP at 20 nm to 81 at 2 μm for a sphere radius of 4 μm and thus limits the maximum achievable momentum.

In reality, a maximum immunomagnetic label density depends not on the densely packed sphere but rather on the present antigens, and association or dissociation constants. Therefore, a complete saturation coverage is not achievable under physiological con-

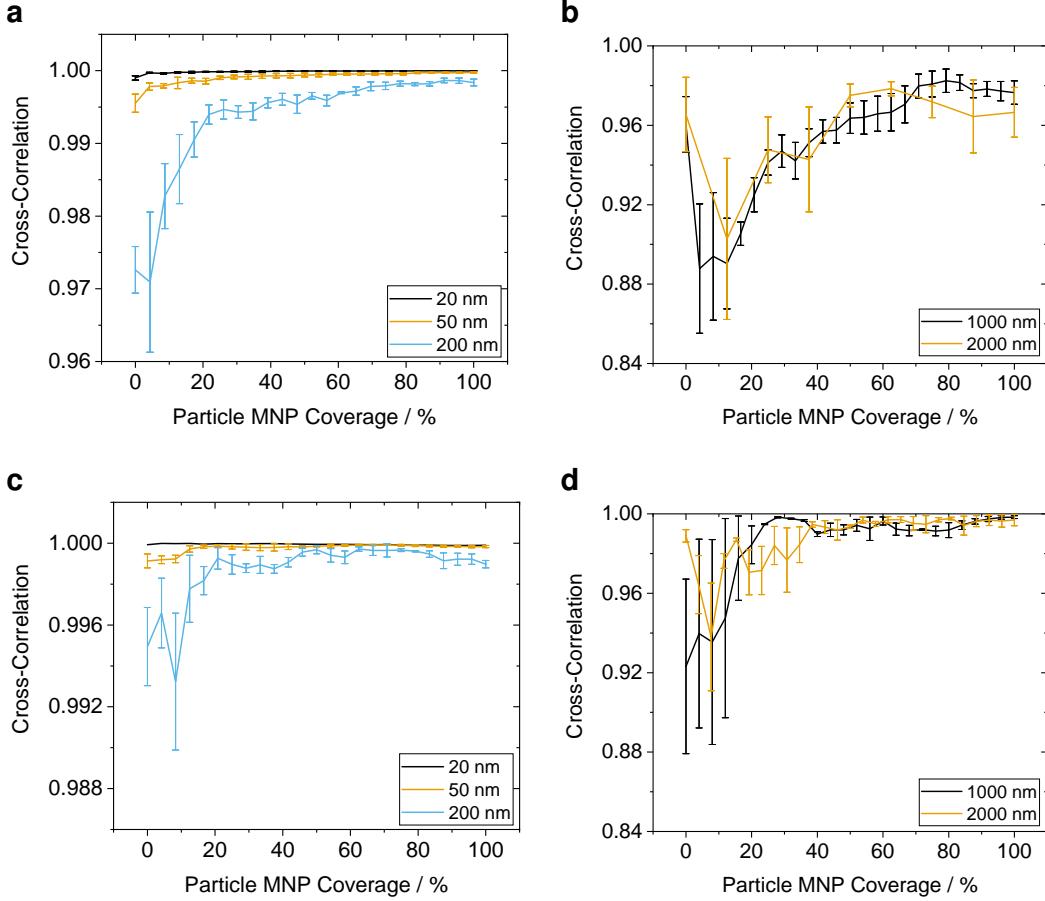


Figure 2: Coverage Dependent Signal Correlation

MNP coverage of a sphere with 4 μm (**a**, **b**) and 8 μm diameter (**c**, **d**) covered by magnetic particles ranging from 20 nm to 2000 nm. A cross-correlation increase which is inversely proportional to the MNP size can be observed.

tions. When using smaller particle sizes, it has to be taken into consideration that a cumulative magnetic moment limited by the number of antigens and the detection limit of the sensing setup.

Also, it can be seen that the correlation approaches a maximum obtainable value for every MNP size. Referenced for both simulated sphere diameters in Fig. 3a, a inverse proportionality between correlation and MNP diameter is visible. The trend in respective maxima can be attributed to variations in the magnetic momentum, and to coverage inhomogeneity.

Inter-sphere compared, maximum correlation shows also a significant deterioration for higher MNP sizes as the particle diameter itself increases. The relative error between both sizes - as computed in Eq. 1.5 - is depicted in Fig. 3b. Assuming a dependency on the available particle surface and subsequently the fraction which can be occupied by MNPs, a quadratic fit seems appropriate.(Eq. 1.6) The fit indicates probably that a cell covered with small MNPs resembles a magnetic dipole located in the center of the cell

while the correlation error becomes nearly constant at great MNPs.

$$\text{Relative Correlation Difference} = 1 - \frac{\max\{\text{Cross-Correlation}(d = 4 \mu\text{m})\}}{\max\{\text{Cross-Correlation}(d = 8 \mu\text{m})\}} \quad 1.5$$

$$\text{Relative Correlation Difference} = -0.385\ 32 d_{\text{MNP}}^2 + 3.345\ 74 d_{\text{MNP}} - 8.496\ 29 \quad 1.6$$

1.1.3. Cell Aggregates

In another simulation, two 200 nm-MNP-covered spheres were attached to each other in differing angles and simulated flowing over the sensor. Signal similarity to a magnetic dipole in the center of a single reference sphere was computed by cross-correlation. As can be observed in Fig. 4, correlation is identical to a single sphere at low coverage, above a threshold magnetization. Here, the randomly distributed particles span an inhomogeneous magnetic field of a larger sphere which cannot be subdivided in the two singular parts.

With growing coverage, each cell starts to form an independent dipole. At occupancies greater than 50 %, the two attached cells are in such superposition. Now, the sensor detects not a single particle as for less coverage but two spatially close particles. Consequentially, the signal is an interference pattern of the two singular events and thus carries small peaks in the center. This is shown schematically in Fig. 1c. In term, this causes a high signal deviation from the reference and a low degree of correlation effectively.

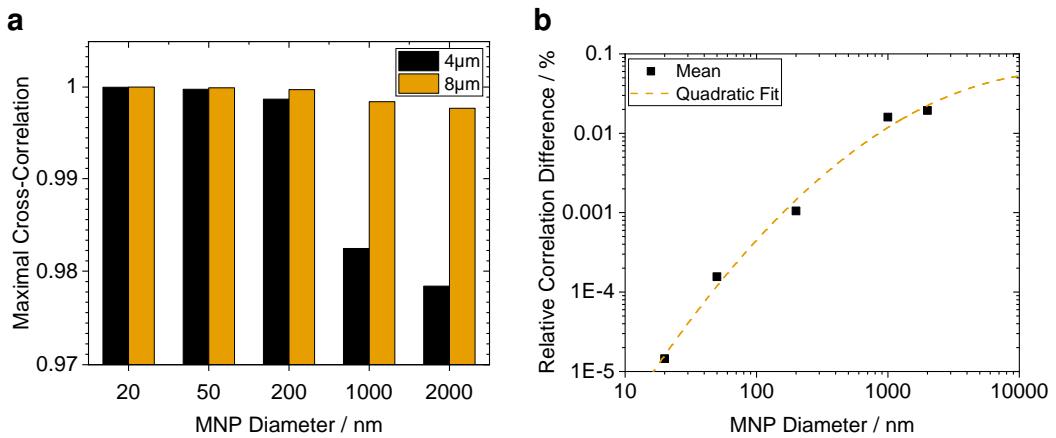


Figure 3: Maximal Cross-Correlation Differences

(a) Mean coverage at 99 % for 4 μm and 8 μm spheres. A negative dependency on the MNP size can be explained by the ratio of magnetic momentum per unit surface and its homogeneous distribution across the whole surface.

(b) Relative correlation error between 4 μm and 8 μm spheres with a quadratic fit. The quadratic behavior could be related to the relative surface area which can occupied by magnetic momentum. (Adj. $R^2 = 0.992\ 09$)

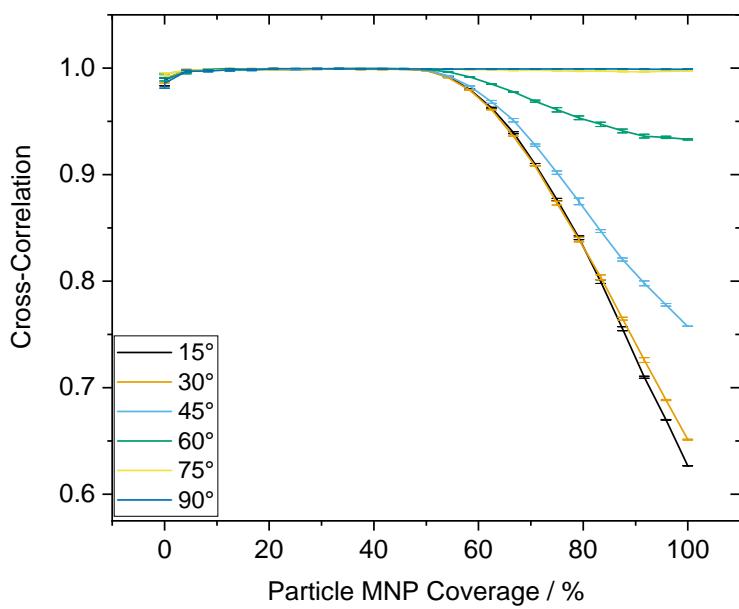


Figure 4: Signal Correlation between Two-Cell Aggregates At Shifting Angles

Two-Sphere aggregates are covered with 200 nm MNPs and simulated flowing over the sensor at differing respective angles. The SEM indicates a difference in cross-correlation of three truly random MNP distributions. For low yaw angles and high coverages, the aggregate's signal reflects rather two single dipoles in superposition than one quite homogeneous dipole. This causes a high signal deviation from the reference and thus a low degree of correlation.

1.2. The MRCyte Simulation Framework

In this work, also an analytical simulation framework that is capable of simulating the synergy of multiple microfluidic effects was developed. The comprehensive framework features magnetic, fluid dynamic and biochemical processes inside the utilized microfluidic channel which act on a particle. Foremost, material parameters were stored inside the “MRCyte” class, which ranged from channel and particle properties to binding and friction constants. Basic velocity, shear and magnetic field computations build the core of the presented program. Additionally, several dimensionless parameters such as the Stokes or Reynolds number (Re) or particle properties can be computed.

With that, simulations of the fluid dynamics that influence a single microbead as well as force-equilibrium computations for the same bead were carried out.

1.2.1. Fluid Fields inside the Microchannel

The simulation framework provided a quantitative generation of the Hagen-Poiseuille flow profile inside the microchannel with the numerical solution of ???. The simulated channel had dimensions (w x h x L) 700 μm x 150 μm x 15 800 μm . The flow rate was adopted to 80 $\mu\text{L min}^{-1}$.¹ Tubing as well as time dependent effects were neglected.

The simulated flow field (\mathbf{u}) for the whole channel cross-section can be observed in Fig. 5a. Due to the no-slip boundary condition, \mathbf{u} is zero on the margin while the maximum of is reached in the geometric center. Mean fluid velocity ($\bar{\mathbf{u}}$) in the channel ensues 12 670.84 $\mu\text{m s}^{-1}$.

Conjointly, computation of the flow gradient in vertical direction and scaling with dynamic viscosity (η) yield the shear stress field.(Fig. 5c) As the curvature of flow field (\mathbf{u}) is zero in the channel center and maximal at the edges, the shear stress reaches highest values symmetrically at the horizontal edges of the channel.² Resulting, the net viscous shear $\tau_{\text{viscous}} = \frac{\partial u}{\partial z}$ cancels out over the whole channel cross-section.

Additionally, \mathbf{u} and viscous stress tensor (τ_{viscous}) acting on a 8 μm diameter bead on the channel bottom were analyzed.(Figs. 5b and 5d) In the proximity of a wall and due to the applied boundary conditions, τ_{viscous} enclosed by the bead surface is non-linear. Thus, the mean fluid velocity exposed to the bead amounts in $\bar{\mathbf{u}}_p = 2241.59 \mu\text{m s}^{-1}$, whereas $\overline{\tau_{\text{viscous}, p}}$ strains with 4.93 dyne cm^{-2} .

¹ in accordance with the experimentally determined value

² Because the horizontal components of the gradients were neglected graceless

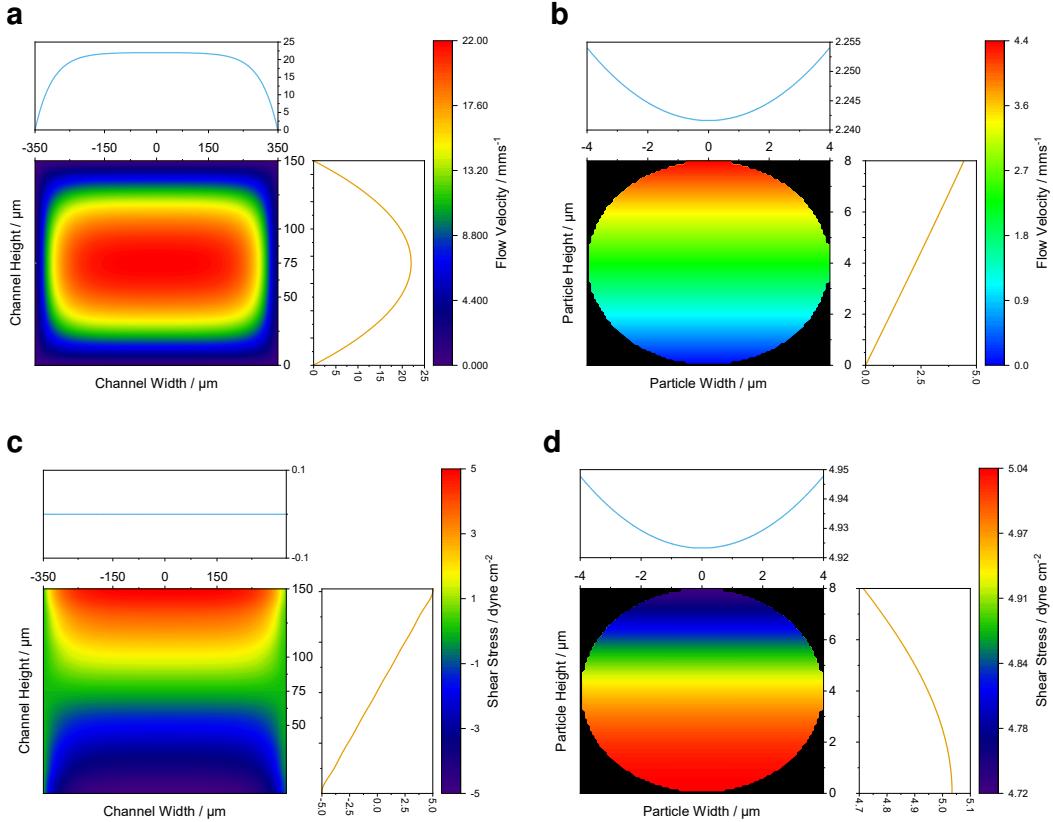


Figure 5: Flow Field and Shear Stress Simulation of the utilized Microchannel

Flow (a) and vertical shear (c) field inside the microchannel with dimensions ($w \times h \times L$) $700 \mu\text{m} \times 150 \mu\text{m} \times 15800 \mu\text{m}$ for a flow rate of $80 \mu\text{L min}^{-1}$ and with neglected tubing effects. The subplots on the right and top side show the mean horizontal and vertical profile in $0 \mu\text{m}$ width and $75 \mu\text{m}$ height, respectively. (vertical: —, horizontal: —) Due to the no-slip condition, the velocity at the walls equals zero and the shear is maximal. The maximum of the Hagen-Poiseuille profile is located in the channel center. Over the cross-section the mean flow velocity \bar{u} equals $12670.83 \mu\text{m s}^{-1}$. Resultingly, the net horizontal viscous shear $\tau_{viscous} = \frac{\partial u}{\partial z}$ cancels out over the whole channel cross-section.

Flow (d) and vertical shear (d) field acting on a $8 \mu\text{m}$ diameter bead on the channel bottom. The mean fluid velocity trapped by the bead profile results in $\bar{u}_p = 2241.59 \mu\text{m s}^{-1}$, whereas the viscous shear strains with $\tau_{viscous} = 4.93 \text{ dyne cm}^{-2}$

1.2.2. Modelling the Force-Equilibrium of a Rolling Bead over a Biofunctionalized Surface

nice intro

With the supplier's parameters of a $8\text{ }\mu\text{m}$ micromer-M bead (micromod Partikeltechnologie GmbH, Rostock) the corrected drag force on a bead on the bottom of the standard utilized microchannel results in 463.65 pN for $80\text{ }\mu\text{L min}^{-1}$. This is computed from the ?? and the correction factor in ??, where the simulated flow field integrated over the particle surface was plugged into.

If the bead was functionalized with biotin under negligence of the differential equations for the association constants, the number of interacting groups would result in the present surface charges. Surface charge density results in $1\text{ }\mu\text{mol g}^{-1}$ of $-\text{COOH}$ (carboxyl) and $-\text{NH}_2$ (amine) beads as of the supplier's data sheet. Hence, a fully saturated bead is covered with 177 500 biotin molecules.

The streptavidin coverage of the channel floor was modeled in excess over the biotin ligands and penetration depth was estimated by the size of several monolayers of protein. As described by Wu and Voldman [1], an approach of 30 nm is a reasonable quantity. In turn, the surfaces were in contact with $1.51\text{ }\mu\text{m}^2$ which constitutes 0.75% of the $8\text{ }\mu\text{m}$ bead surface. This reveals that 1329 biotin molecules can interact with the floor. A summation of the protein-bond force ($\mathbf{F}_{\text{protein}}$) at 5 pN to 150 pN per streptavidin-biotin bond yields the resulting adhesion force with a magnitude of 6.7 nN to 199 nN .[2]

The binding force is in the same range as the perpendicular magnetophoretic force caused by the permanent magnet under the sensor chip ($\nabla\mathbf{B} = 10\text{ T m}^{-1}$) as well as by the nickel-iron chevron structures on the chip ($\nabla\mathbf{B} \approx 5\text{ kT m}^{-1}$). Clearly, in the near-field approximation the nickel-iron structures dominate magnetic force (\mathbf{F}_{mag}) (??). With the manufacturer given saturation momentum of one particle (1.12 pA m^2), the magnetic attraction force eventuates in 5.6 nN in the magnetophoresis section of the channel.

$$\mathbf{F}_{\parallel} = \mathbf{F}_{\text{drag}} - C_{\text{rr}} \cdot (\mathbf{F}_{\text{mag}} + \mathbf{F}_{\text{protein}} + \mathbf{F}_{\text{grav}} - \mathbf{F}_{\text{shear}}) \quad 2.7$$

$$C_{\text{rr}} = \sqrt{\frac{z}{d}} = \sqrt{\frac{30\text{ nm}}{8\text{ }\mu\text{m}}} = 0.0612 \quad 2.8$$

In order to merge this analytic force balance, all remaining forces have to be projected into the direction of Stoke's drag force (\mathbf{F}_{drag}).(Eq. 2.7) This is achieved by the introduction of a rolling resistance factor (C_{rr}) for a perfectly elastic surface.(Eq. 2.8) In a first order approximation, the factor depends only on the approach (z) and the bead diameter (d). However, scientific literature about the rolling resistance of microbeads on microfluidic or protein covered surfaces does not exist yet to confirm this macroscopic factor for the microscale.

Scaling all orthogonal forces to the Stoke's drag force with C_{rr} yields a net positive result (154.08 pN) for an unfunctionalized surface ($F_{protein} \doteq 0$) which indicates a rolling motion in flow direction. Notwithstanding, above a critical interaction number of 503 to 16 biotin-streptavidin bonds - for the respective release forces of 5 pN to 150 pN per linkage - the particle resists Stoke's drag force and adheres to the surface.

This behavior will be exploited in further measurements for "bead loss experiments" in order to measure a concentration difference with different degrees of biotinylated beads.

1.3. Reference Bead Surface Functionalization

After simulation of their respective coverages, biotin was titrated on 8 μm reference beads with two different surface terminations in order to selectively bind MNPs with the counter-agent streptavidin to the surface. First, amine-microbeads were modified by sulfo-NHS-biotin. Second, carboxyl-beads were coated by amine-PEG₂-biotin via EDC-NHS-activation. On the same beads Anti-IgG1-PE antibodies were titrated after the same coupling chemistry.

Subsequently, biotin-coated beads were analyzed in the flow cytometer in the by staining with Atto-488 (Ex: 500 nm, Em: 520 nm) coupled streptavidin. The antibody was modified with phycoerythrin (PE) and measured at 488 nm excitation and 585 nm emission wavelength. The gating was standardized by the strategy found in ??, ?? . Subsequently, the median fluorescence intensity (MFI) was computed and fitted with a sigmoidal Hill-function.(??) Stability of carboxylated and aminated beads and subsequently their respective modification protocols was evaluated for 12 days.

1.3.1. Amine-Surface Biotinylation

As first approach, polystyrene copolymer microbeads with 8 μm diameter were functionalized by (sulfo-)NHS-biotin after a standard protocol from Thermo Fisher Scientific and micromod. A titration of the biotin reactant yielded a varying surface coverage as shown in Fig. 7a. During this one-pot-reaction, the water-soluble sulfo-NHS-biotin forms an R₁—CONH—R₂ (carboxamide) linkage with the primary amine and 1-hydroxy-2,5-dioxopyrrolidine-3-sulfonate splits off as byproduct.

As can be seen from the SEM error-bars from the plot 7a, which were obtained from three true biological replicates, this process is highly reproducible. Therefore, surface coverage in different grades of biotinylation could be incurred accurately with a adjusted coefficient of determination (adj. R^2) of 0.981 for the resulting Hill fit.

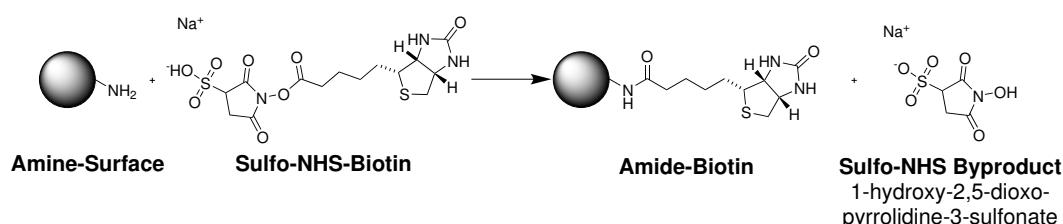


Figure 6: Amine Bead Modification with Sulfo-NHS-Biotin

An amine terminated bead brought into reaction with sulfo-NHS-biotin. Both form an amide linkage and bind biotin covalently to the surface. As byproduct the sulfo-NHS-ester splits off.

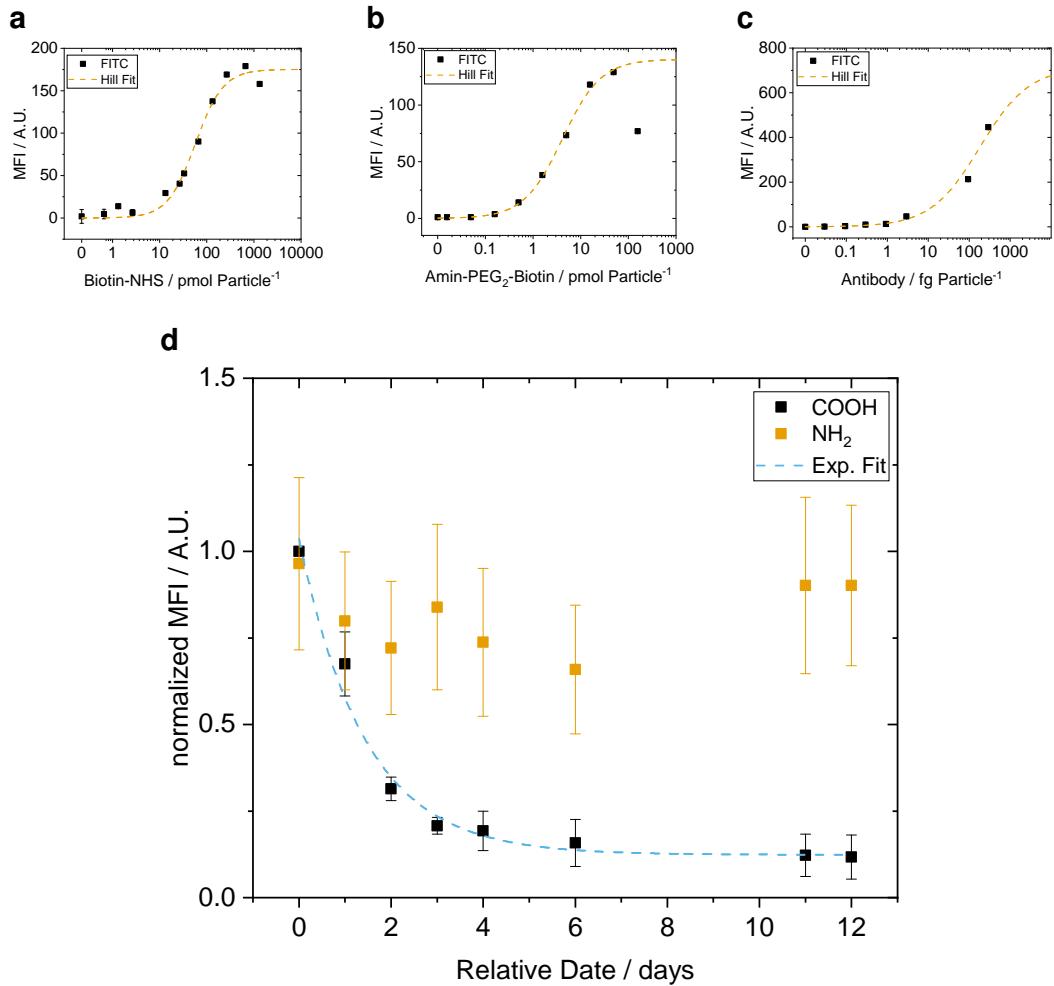


Figure 7: Titration of Biofunctional Molecules on 8 μm Particles

Titration curves of NHS-biotin (a), Amin-PEG₂-Biotin (b), and Anti-IgG1 (c) with their respective Hill fits. The corresponding fit parameters as well as the goodness factor are shown in Table 2a. (d) Stability analysis of functionalized carboxyl and amine beads over 12 days. The carboxylate particles show an exponential decrease with a half-life of 1.43 days as determined by the exponential fit. The respective parameters are shown in Table 2b.

a				b		
Param.	Hill 7a	Hill 7b	Hill 7c	Param.	Exp. 7d	
V_{max}	175.216 19	140.391 53	713.836 43	A	0.912 63	
k	57.367 13	4.126 61	182.830 11	τ_{decay}	1.425 57	
n	1.474 88	1.074 93	0.724 58	y_0	0.123 69	
Adj. R^2	0.981 21	0.997 22	0.992 26	Adj. R^2	0.966 55	

Table 2: Fit Parameters of Biotinylation

(a) Coefficients for the Hill fits in Figs. 7a to 7c (b) Exponential fit coefficients for the stability analysis in Fig. 7d

Carboxylate-Surface Functionalization

In a second approach, particles with opposite partial surface charge, mediated through carboxyl groups, have been functionalized. In turn, particles were pre-activated in 3-(Ethyliminomethyleneamino)-N,N-dimethylpropan-1-amine (EDC) and 1-Hydroxy-2,5-pyrrolidinedione (NHS) in 2-(N-morpholino)ethanesulfonic acid (MES)/MES buffer with Tween 20 (MEST) buffer. There are two distinct reasons for the usage of MES based buffers rather than phosphate buffered saline (PBS) or MACS running buffer (MACS). First, EDC has its reactive maximum at pH 5 to 6. Second, buffers containing primary amines (TRIS / glycine) or carboxyls (acetate / citrate) will quench the reaction and therefore limit the efficacy.

Afterwards, the beads were washed carefully and incubated with amine-PEG₂-biotin. Here, poly(oxyethylene) (PEG) indicates a hydrophilic spacer arm between both functional groups and in this case has length of a two units. The full functionalization procedure is explained in more detail in ??.

As shown in Fig. 7b, particles were functionalized equally compared to carboxamide surfaces. However, the stability of carboxyl particles yields a half-life of 1.43 days in a continuous measurement over 12 days with a subsequent exponential fit. Additionally, both procedures show an outlier at high concentrations which could not be explained during the course of this thesis.

Third, carboxylated particles have been also functionalized with the Anti-IgG1-PE antibody. Again, a Hill-shaped titration curve was achieved, but due to the costly reagent a saturated surface coverage was not reached. (Fig. 7c)

Therefor, the fit curve has to be interpreted cautiously. Although it converged and represents the data with an adj. R^2 of 99.2 %, the goodness of fit determined by the reduced χ^2 statistic results in a value of 278.1 which indicates an underestimation of the error variance.

1.4. Concentration Measurements in MRCyte

Driving factor for the concentration measurement is the absolute count of immunomagnetically labeled cells in diluted or whole blood which is not possible in today's optics-based devices due to the excess of red blood cells (RBCs).[3] Therefore, with the in ?? described deterministic approach of cell focusing for subsequent magnetic detection, absolute concentrations of magnetic reference beads were attempted to measure.

Beads with acrylate surface were pumped through a microfluidic channel with a permanent magnet underneath. The magnet drew every magnetic particle to the ground, where they were focused on the sensor bridge and subsequently measured there. From the received signal several parameters such as peak amplitudes, locations, zero-crossings, and relative distances between each other were computed.(Fig. 8) Especially for the concentration measurement a correct detection of bead signals from the noisy stream or from a superposition of multiple, simultaneously measured particles was critical. The related error sources and countermeasures will be elaborated in Sec. 1.4.1.

By measuring the absolute concentration with a commercially available flow cytometer (MacQuant 10, Miltenyi), a reference bead count was established. In a pre-test, beads were taken directly from the microcentrifuge tube, after pumping through a syringe, and after pumping through a syringe with 10 cm of connected through tubing (ID 0.5 mm, RS Chemicals). Afterwards, they were counted in the flow cytometer in equal volumes. Additionally, two different buffers - MACS and PBS - and two different surface terminations were used. Both buffers are based on phosphate buffered saline (PBS). Notwithstanding MACS contains 2,2',2'',2'''-(ethane-1,2-diyl)dinitrilo)tetraacetic acid (EDTA) as chelator for divalent ions, Tween 20 - a non-ionic surfactant -, and an azide-based stabilizer. Hence, the wetting of surfaces and the electrostatic interactions of these buffers differ. The same properties were varied on the bead surface by choosing acrylate- and biotin-terminated beads.

In Fig. 9, a trend (without statistical confidence) can be observed that shows a decrease in particle counts after every additional surface which beads could potentially interact with. In term, a correct count in absolute numbers seems out of range. However, a calibration of the system with the flow profile inside the channel to compensate for losses subjected to connectors and magnetic enrichment structures was carried out successfully.

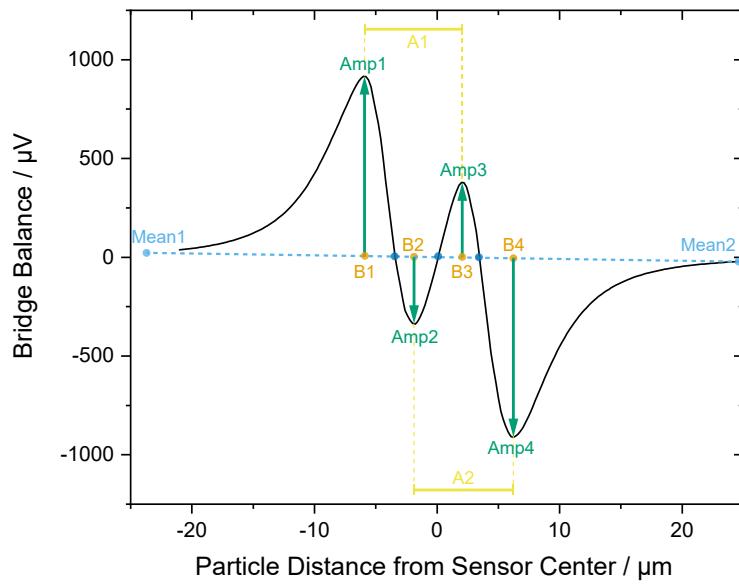


Figure 8: Example Signal of Magnetic Measurement

Signals generated from the Wheatstone bridge sensor setup feature a certain shape which allows for several measures. In case the overall signal stream carries a constant or linear offset, it is scaled to the means before and after the detected peak pattern. (Mean1, Mean2) The x- and y-positions of each peak are denominated by B1-4 and Amp1-4, respectively. The crossings of the signal through the linear connection of both means are denominated by n1-3 (in the figure by ●). Further, the difference between the equally oriented peaks B3-B1 and B4-B2 give a measure for the homogeneous movement of the measured object and are called A1 and A2 each. From these values the overall velocity v can be approximated because the GMR bridge distance (d_{GMR}) and sampling frequency (f_s) is fixed precisely. Analogously, the magnetic diameter of a dipole is computed by the mean of the differences B2-B1 and B4-B3.

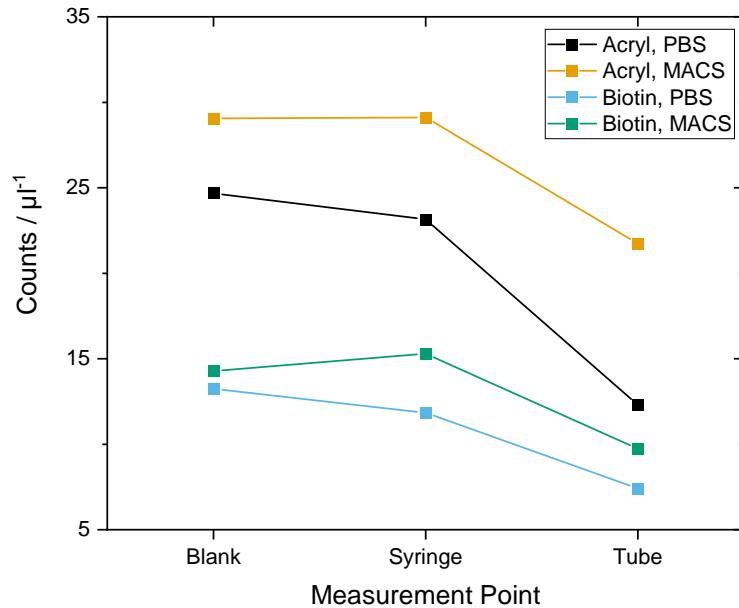


Figure 9: Bead Loss Evaluation in Connectors

Bead concentrations measured in equal volumes in the flow cytometer after being pumped through a syringe or a syringe with connected tubing. The blank sample was measured directly from the stock solution. Additionally, electrostatic and surface tension related effects were resolved by the usage of different buffers and bead surfaces.

1.4.1. Measurement Error Sources and Calibration of Flow Field

In order to account for the bead losses due to the tubing connectors, the Hagen-Poiseuille flow profile, and magnetophoretic enrichment structures, the measured bead concentration was corrected in two different approaches.(Eq. 4.9) On the one hand side, the typical assay correction to the ground truth by a constant linear fit correction factor (C_{const}) computed from the blank population was established. On the other side, a velocity correction factor (C_{velocity}) compared the mean fluid velocity (\bar{u}) to the bead velocity (v_c).

$$c_{\text{beads, expected}} = c_{\text{beads, measured}} \cdot C \quad 4.9$$

The C_{const} relates a reference count in the optical flow cytometer to the measurement in the magnetic flow cytometer.(Eq. 4.10) Equally-adjusted bead concentrations in the samples allow for a correction to the reference system. However, for an assay usage the initial concentration of beads either has to be known precisely or has to be irrelevant, for example in regards of a standardized measurement procedure. Besides, C_{const} provides a reliable and generalizable option for correction.

$$C_{\text{const}} = \frac{c_{\text{beads, standard procedure}}}{c_{\text{beads, MRCyte}}} \quad 4.10$$

$$v_c = 2 d_{\text{gmr}} \frac{f_s}{n_3 - n_1} \quad 4.11$$

$$C_{\text{velocity}} = \frac{\bar{u}}{v_c} = \frac{Q}{A \cdot v_c} \quad 4.12$$

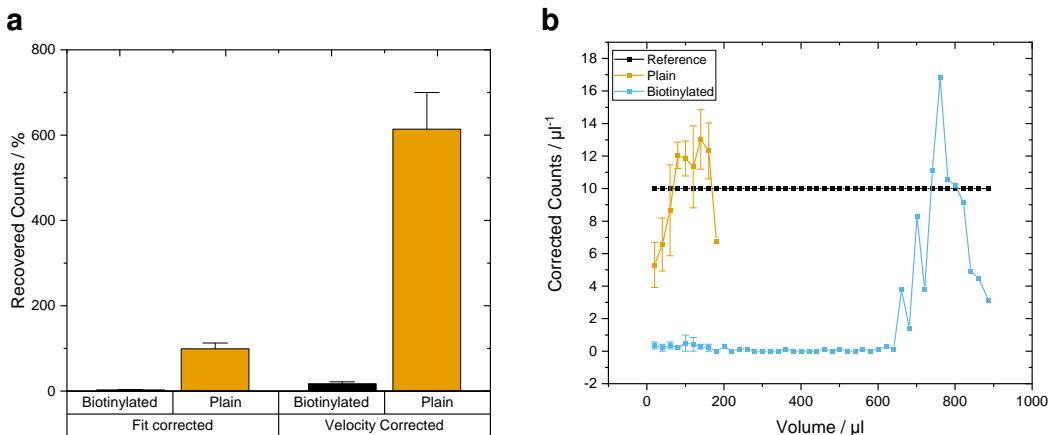


Figure 10: Error Sources in Concentration Measurements

(a) Robustness evaluation of the both correction factors C_{const} and C_{velocity} for protein coated surfaces. The mean and SEM of plain and biotinylated measurements show a high deviation from physically reasonable expectations when corrected for the velocity (right). In contrast, C_{const} can intrinsically correct only well below 100 %. (b) Mean and SEM of a fit-corrected bead capture experiment with several error sources. Initially, the magnetophoretic structures have to be filled and thus decrease the plain count for the first 100 μL . (—) Additionally, the high deviation offsets the correction factor so that the stable measurement from 100 μL to 200 μL lies now above the ideal reference. In contrast, the biotinylated beads are captured by the surface functionalization and hence a very low concentration is measured. However, a steep rise with pulsations can be observed when the surface is saturated with beads and the particles begin to flow over the sensor in bursts. (—) The abrupt decline to the end of the measured volume is most probably related to sedimentation effects inside the emptying syringe.

The C_{velocity} relates the effective particle velocity to the total fluid velocity in order to eradicate flow profile provoked effects.(Eq. 4.12) Whereas \bar{v} was determined by flow rate (Q) through a cross-sectional area (A) of the channel, v_c was analyzed from the measured signal stream. Here, the intrinsic GMR bridge distance (d_{GMR}) was divided by the time difference where the bead passed exactly over a half bridge with known distance.(Eq. 4.11) These specific timepoints are visible as dimensionless zero-crossings n_1 and n_3 in the signal and can be converted by scaling with the sampling frequency (f_s) into the time domain.(Fig. 11a) However, if the bead velocity is not solely dependent on

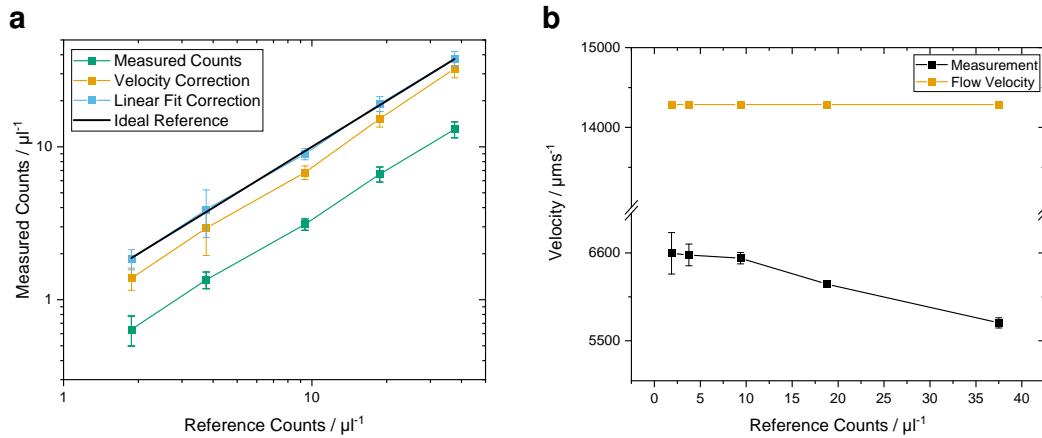


Figure 11: Absolute Concentration Measurements

(a) Mean and standard deviation of the concentration measurement from three independent measurements. The uncorrected measurement shows a highly reproducible and linear count over the dynamic range of almost two decades. (—■—) The ideal reference from the flow cytometer is depicted in (—). Correction with C_{velocity} (—□—) yields at a factor of 2.261 09 which is 21.7 % more imprecise than C_{const} at $2.888\,33 \pm 0.080\,75$.(—△—) (b) Mean and SEM of the v_c estimation from the signal. For higher concentrations, the measured velocity becomes inaccurate thus distorts the correction factor.

fluid dynamic effects - especially in the light of surface functionalizations - C_{velocity} can not be applied to experiments robustly. This is depicted in a sample experiment with a protein covered surface in Fig. 11a. By definition, the C_{const} can not be well above than 100 % whereas the count correction by C_{velocity} differs by 600 % through variations in the velocity measurement.(Fig. 10a)

An adaptation of these corrections to real measurements are depicted in Fig. 11. In a measurement where 300 μL were dispensed into the magnetic flow cytometer with a defined particle concentration the counts were analyzed and corrected according to above. This time, the channel had a cross-section of 700 $\mu\text{m} \times 50 \mu\text{m}$ (w x h) and flow rate (Q) was set to 30 $\mu\text{L min}^{-1}$.

Apart from a reproducible count over the dynamic range of almost two decades, both correction factors ameliorated the present data. C_{const} amounted in an optimum 2.89 ± 0.08 while C_{velocity} centered around a mean of 2.26. Consequently, the velocity correction was misguided by 21.7 % for the advantage of requiring no *a priori* knowledge about the measurement.

Another peculiarity of C_{velocity} can be observed in Fig. 11b. While the analyzed velocity

surface stability wegkreissen
von streptavidin

is stable for less than $10 \mu\text{L}^{-1}$, a linear decrease is visible for higher concentrations. This is a consequence when signals of beads start overlapping if these are flowing over the sensor in a close vicinity. Hence, the disturbed signal sensitizes the parameter reconstruction to errors such as false peak-identification.

1.4.2. Concentration Measurement in Diluted Whole Blood

The same concentration measurements from before were now carried out in whole blood samples. Here, the reference count can be attained only below the experimentally determined critical concentration around $10 \mu\text{L}^{-1}$. (Fig. 12) An insignificant discrepancy could be perceived in different volumetric blood to buffer dilutions of 1:1 and 1:20, respectively. This provides evidence for the measurement's independence from the blood concentration in buffer.

However, a significant difference between counts was discovered between a $150 \mu\text{m}$ high channel, where C_{velocity} can be determined accurately, and a $50 \mu\text{m}$ high channel, where the correction yields a great error to the reference. This may be subjected to an increased probability of collisions from beads with blood cells and hence a decreased velocity which in turn leads to a higher correction factor. Another explanation approach could be the transition from *Newtonian* to *Non-Newtonian* fluid dynamics in smaller cross-sections, which could likewise be influenced by the Fåræus effects mentioned in ??.

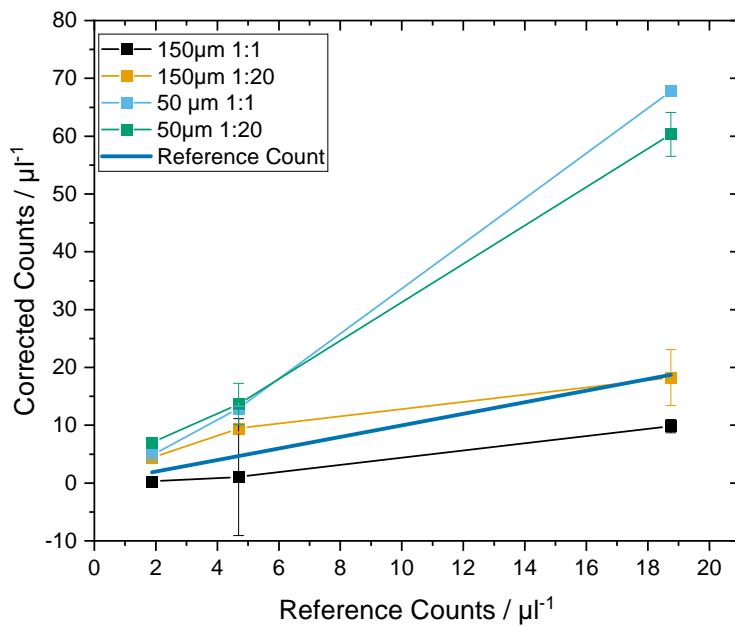


Figure 12: Absolute Concentration Measurement in Blood Samples Under Varying Channel Height
Velocity corrected concentration measurements for two different blood dilutions and channel heights. While C_{velocity} works for high channels in all tested concentrations, it does not work for high concentrations in $50 \mu\text{m}$ high channels. This is probably a result of bead-cell collisions and the resulting path interruption.

1.4.3. Surface Magnetization of Biofunctionalized Beads

Here, the previously surface-modified polystyrene beads were magnetized with MNPs and counted in the magnetic flow cytometer. Originally, four different magnetic nanoparticles have been tested. Albeit, both nanomag-D-spio 100 nm (micromod Partikeltechnologie GmbH, Rostock) and dynabeads MyOne Streptavidin C1 1 μm (ThermoFisher scientific, Waltham, USA) showed inconclusive results and are omitted in the latter.

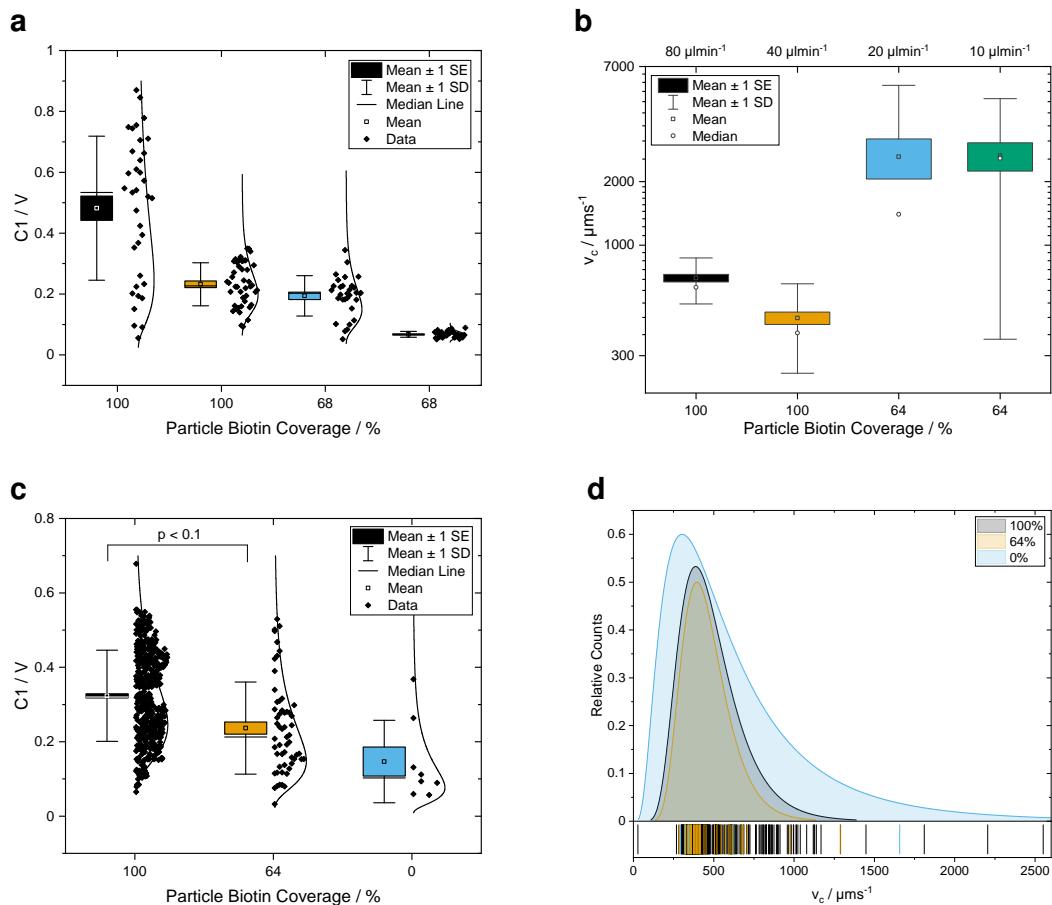


Figure 13: Bead Coverage Assay with Magnetic Streptavidin Nanoparticles

Magnetic flow cytometry data from 8 μm polystyrene sphere which were biotinylated in different degrees and subsequently coated with BNF-Dextran-redF-streptavidin 100 nm MNPs (a,b) or SV0050 50 nm streptavidin MNPs (c,d). (a) Signal amplitude of the counts with various flow rates 1. 80 $\mu\text{L min}^{-1}$ 2. 40 $\mu\text{L min}^{-1}$ 3. 20 $\mu\text{L min}^{-1}$ 4. 10 $\mu\text{L min}^{-1}$ (b) Reconstructed velocities of the respective populations. The 100 % biotinylation shows plausible velocities, whereas the 68 % sample can either be considered as noisy background or very weakly magnetized particles.(c) Signal amplitude with 80 $\mu\text{L min}^{-1}$. A correlation between biotinylation degree and magnetic momentum can be assumed at a p-value $p < 0.1$ (d) Velocity distributions of the samples. As postulated, the mean velocities do not differ, moreover, are enveloped by the blank sample.

First, BNF-Dextran-redF-streptavidin 100 nm MNPs (micromod Partikeltechnologie GmbH, Rostock) were attached to the non-magnetic beads after the protocol in ???. Then, the magnetizability was examined qualitatively in a magnet stand. Particles were considered “magnetically labeled” if a pellet was visible after 10 min. Afterwards, the concentrations were measured with the optical flow cytometer and adjusted to $10 \pm 1 \mu\text{L}^{-1}$

accordingly.

The subsequent measurement of 300 μL in the magnetic flow cytometer is shown in Fig. 13. In Fig. 13a the peak difference C1 (= Amp2 - Amp1, Fig. 8) is presented against the biotinylation degree. Independent experiments of 100 % and 68 % biotinylation show a certain amplitude difference. The respective fluid and particle velocities in Fig. 13b provide an explanation for this behavior. The fluid velocity had to be adapted during the course of the experiments from $80 \mu\text{L min}^{-1}$ to $10 \mu\text{L min}^{-1}$ in order to receive stable counts.

Keeping this in mind leads to the fact that - although only two biotinylation coverages were measured - four distinct magnetizations are represented here. Both 100 % samples show slow but plausible velocities and can therefore be correlated with differing magnetic momentum. Beads with 68 % show an exceptional velocity and can hence either be considered as noisy background or a very weakly magnetized particles that are not pulled to the channel bottom completely. This could also explain the decline in C1 which is also a measure for magnetic dipole moment.

Second, SV0050 50 nm streptavidin MNPs (OceanNanotech, San Diego, USA) were deposited after the equal procedure on 8 μm polystyrene beads. These experiments show the expected result of declining peak amplitude at lower biotinylation and constant velocity throughout. (Figs. 13d and 13e) At a p-value smaller than 10 %, the high populations differ significantly from each other while the log-normal fits match the histograms with a adj. R^2 of 0.94.

Two hypotheses can be drawn now from this result. On the one side, OceanNanotech MNPs could posses more magnetic momentum per particle, which favors the robust measurement. On the other side, streptavidin or MNP size could influence the saturation of all available biotin sites of the particle.

1.4.4. Differential Counting Setup

With regard to the necessity of correction factors in order to resolve the ground truth of a concentration measurement, a different magnetic flow cytometer setup has been evaluated. Here, two fully assembled sensors with printed circuit boards (PCBs) were stacked on top of each other and connected in series which was expected to yield two beneficial effects.

First, one permanent magnet underneath the lower sensor chip should supply both chips with enough gradient field to pull beads to the respective channel bottom. Second, simultaneous signal acquisition should act on the one side as a time-of-flight detector with a relatively long transport distance and on the other side, a differential concentration measurement was envisioned between both chips. Therefor, the hypothetical optimum parameter set could be reached when the relative concentration measurement yielded

identity:

$$\frac{c_{\text{top sensor}}}{c_{\text{bottom sensor}}} = 1 \quad 4.13$$

The system comprises of two separately assembled sensor PCBs with nylon spacers between the positional screws.(Fig. 14) A 3 mm hole was drilled into the top PCB carefully between the strip lines to minimize the tubing length from the top chip outlet to the bottom chip inlet. After the build-up of the differential counting system,³ the hysteresis of both sensor elements was maximized for sensitivity and the concentration was measured against a reference from the optical flow cytometer.

Sensitivity Calibration

Initially, the permanent magnet was adjusted in three linear directions in order to maximize the magnetic sensitivity of both Wheatstone bridges. Schematically, this can be envisioned as a placement of the whole top as well as the whole bottom bridge configuration into the operational range of the magnet, which is indicated by the blue triangle.

Then, the hysteresis of both outermost and one centered sensor was optimized for their full coverage and sensitivity. The recorded values for a sole optimization of the top or bottom sensor array with a height variation of the utilized nylon spacers are shown in Figs. 14b and 14c. Apparently, the optimized sensor exhibits a monotonously higher magneto-resistive effect (MR effect) than the disregarded bridge. Nevertheless, the complete sensor setup features a MR effect well above 7.2% which is acceptable both for measuring immunomagnetically labeled cells and magnetic microbeads. Furthermore, the homogeneity of the interspersing magnetic field can be monitored in the triangular shape of every acquired curve in the sensitivity plots. As the field vectors of magnetic flux density (\mathbf{B}) start to disperse at the outsides of the illustrated blue area in Fig. 14c, the outer sensor regions are not located completely inside a homogeneous gradient field and get distracted by non-perpendicular field components.Helou [4] Additionally, no influence of any spacer height could be discovered. The difference in MR effect varied insignificantly in $0.14 \pm 0.60\%$ of MR effect.

³ as described in ??

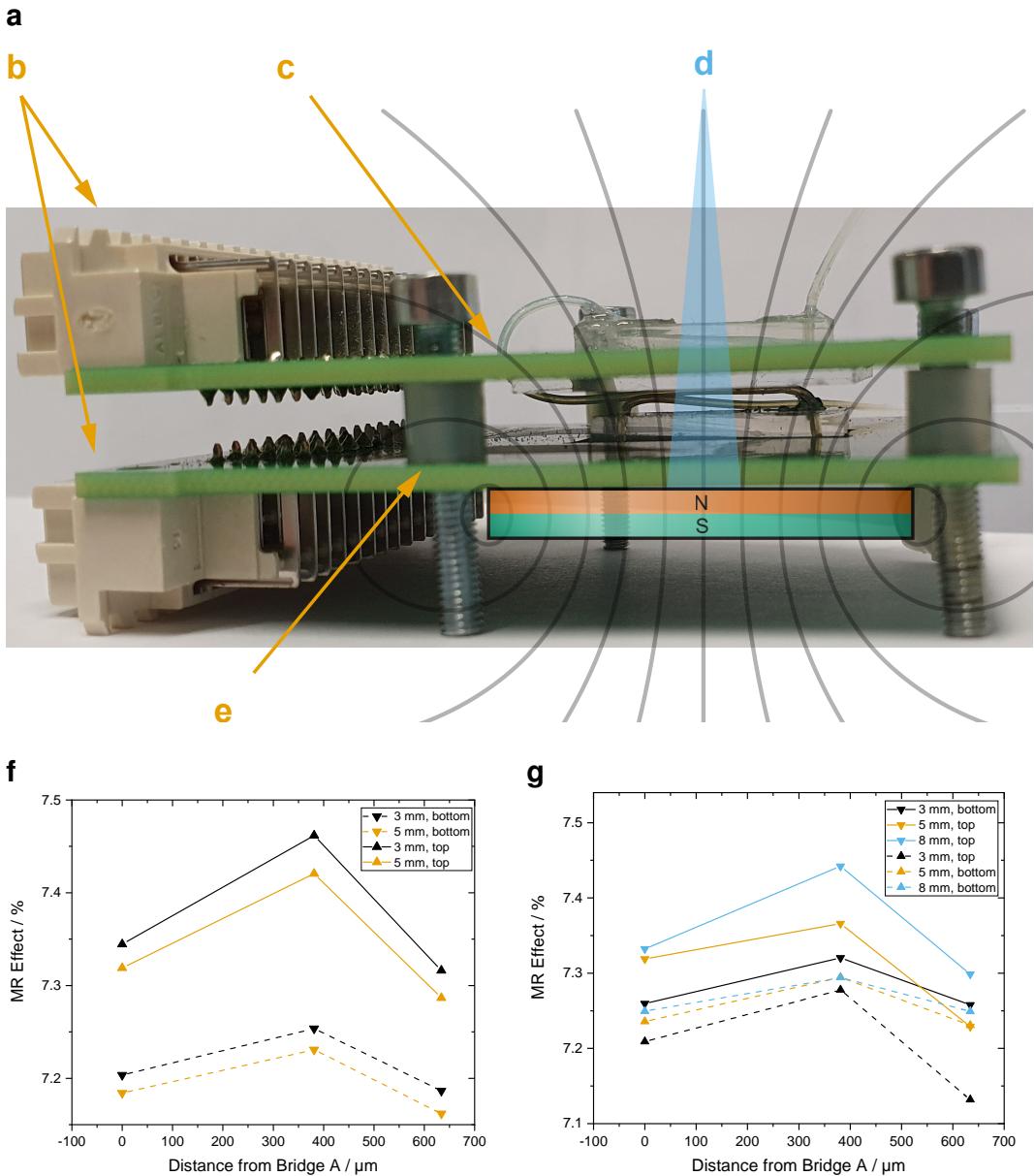


Figure 14: Hysteresis Calibration for Stacked PCB Setup

(a) Differential measurement setup: the system comprises of two separately assembled sensor PCBs (**b**) with nylon spacers (**e**) between the positional screws. (**f**) A hole with 3 mm diameter was drilled between the strip lines to connect the top chip outlet to the bottom chip inlet and minimize tubing length thereby. Schematically, a permanent magnet is placed below the bottom PCB. (**d**) The field line density respectively the area with negligibly differing field vectors is shown in the blue triangle. Because the adjustment is always carried out for a single bridge at once, this causes a systematic error.

The magneto-resistive effects of both sensors, calculated from their hystereses, are depicted for top and bottom sensors, respectively. Whereas the hysteresis was optimized for the centered sensor bridge (D or E) on the top sensor in (**f**), it was optimized for the bottom sensor in (**g**). Additionally, the height of the nylon spacers was varied from 3 mm to 8 mm but showed no statistical correlation.

Concentration Measurement in Buffer Solution

Due to the single utilized permanent magnet for two sensors, another field related issue arose during concentration measurements. This requires a step back inside the general functionality of the magnetophoretic enrichment:

Any bead flowing at an arbitrary position in the microchannel experiences a negative magnetic force by the gradient of the magnetic flux density caused by the permanent magnet which pulls perpendicular to the bottom surface. Upon coming in close proximity to the lower boundary, a gradient provoked by the lithographic nickel-iron structures starts to gain strength with the third power of the distance. This causes the beads to attach to the bottom firmly and enforces their rolling behavior. However, a fragile equilibrium between magnetophoresis and drag has to be maintained slightly in favor of Stoke's drag force for a continuous rolling motion. This ideal state is a narrow space in between two boundary cases.

First, if drag force exceeds magnetophoresis, the beads will not migrate to the channel bottom in the top chip and hence result in a lower concentration measured on top. Second, if magnetophoresis outnumbers drag so that particles flow steadily in the top channel, the beads in the lower channel will stop rolling and adhere statically. In effect, the lower concentration measurement is compromised.

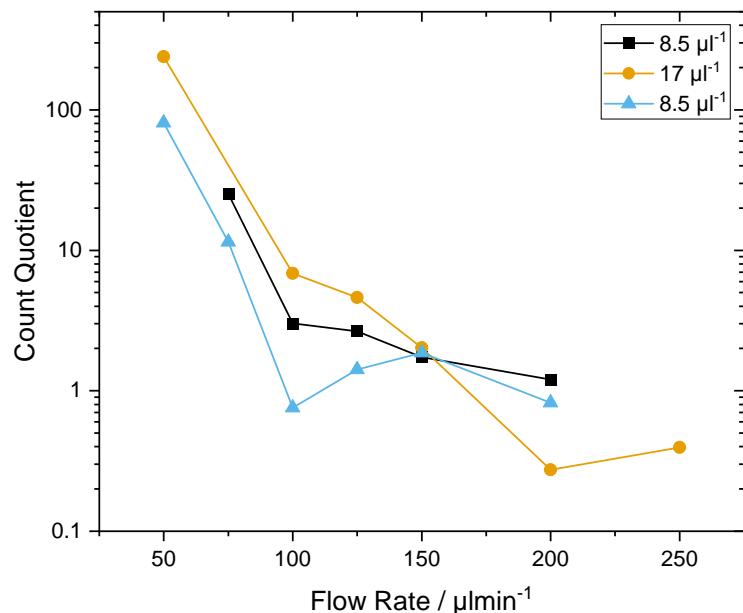


Figure 15: Optimal Differential Counting Flow Rate
Losses in different buffers and bead surfaces.

In order to find the flow rate for the optimal ratio between drag and magnetic force, measurements were performed with bead concentrations ranging from $6.5 \mu\text{L}^{-1}$ to $34 \mu\text{L}^{-1}$.⁴

⁴ The raw data is shown in ??

The quotient of both measured concentration was determined after Eq. 4.13 and plotted in Fig. 15. Although, the optimal quotient value would have been 1 theoretically, an ideal overlap was found for a flow rate of $150 \mu\text{L min}^{-1}$. However, the difficulties in assembly and operation inhibited a further usage in experiments.

1.5. Surface Modification and Biofunctionalization of the Sensor Chip Substrate

In consideration of the problems of surface instability analyzed in Sec. 1.4.1 and to avoid additional uncertainties in the experimental validation of the model from Sec. 1.2.2, a covalent functionalization of the sensor surface with neutravidin was carried out. First, a plate reader experiment for a qualitative statement about the shear-force stability of protein adsorption was performed. Second, different functionalization approaches with a mixture from hydrogen peroxide with excess of sulfuric acid (piranha) and hydrofluoric acid (HF) were tested with pure glass, poly(dimethylsiloxane) (PDMS), and eventually silicon nitride (Si_3N_4). Third the validation of these procedures was limited to indirect measurements such as tensiometry, fluorescence microscopy, and quantitative bead capture assay, as sophisticated chemical analyses were hardly available.

1.5.1. Physisorption

In order to quantify the adsorption stability for fluorescently-labeled, physisorbed streptavidin molecules, sensor chips were cut into 10 mm^2 pieces and glued to the bottom of a 96-well plate. Subsequently, they were equilibrated with PBS and incubated with 1 mg mL^{-1} over night. Each measurement was corrected for a blank substrate as well as the negative control with plain PBS buffer and normalized subsequently.

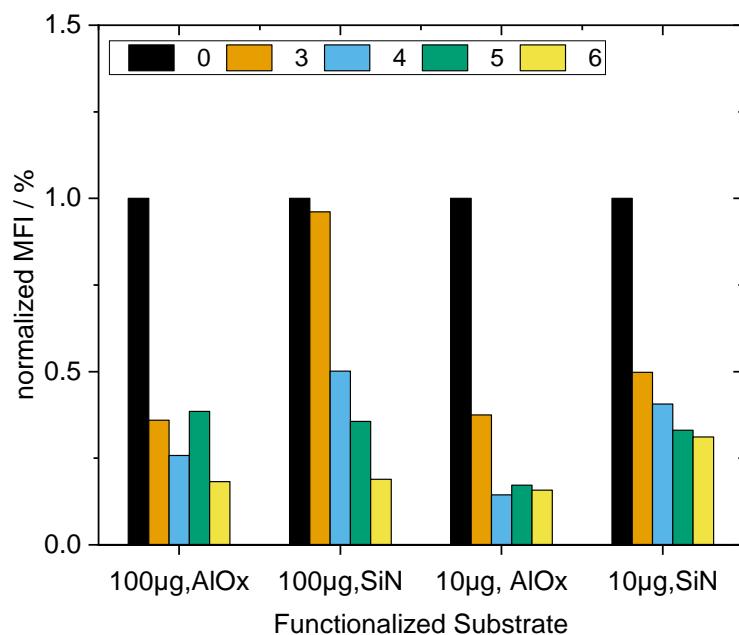


Figure 16: Surface Adsorption Stability of Neutravidin on Si_3N_4 and Aluminium oxide (Al_2O_3)

Plate reader measurement with $3 \text{ mm} \times 3 \text{ mm}$ Si_3N_4 and Al_2O_3 samples which were incubated with $100 \mu\text{g}$ and $10 \mu\text{g}$ streptavidin-atto488, respectively. The samples were subsequently washed with $200 \mu\text{L}$ PBS carefully. Fluorescence intensities were of corrected with a blank substrate, the autofluorescence of PBS, and normalized eventually. Every surface reaches a mean fluorescence level of 28 % after few washing steps.

Every sample in the plate reader showed a significant surface decrease to a mean level

of 28 % from the original fluorescence.(Fig. 16) Whereas proteins desorbed from both crystals after the first washing steps equally, Si_3N_4 outperformed Al_2O_3 as more stable in steady state. (34.8 % vs. 27.5 %) However, no quantitative hypothesis could be formulated by these numbers due to the nature of their indirect measurement. Hence, the protein activity which is the crucial quantity for any bead rolling remains questionable. Nevertheless, a qualitative proposition is strongly confirmed that unspecifically adsorbed proteins are removed from any of both surfaces rapidly.

1.5.2. Evaluation of the Covalent Biofunctionalization with Optical Methods

Now, the results of several covalent surface modification procedures with various substrates are presented. Foremost, glass was used as main carrier material. On glass established protocols were then brought onto PDMS and Si_3N_4 chips. As main functionalization protocol, an activation in 7:1 piranha was carried out for 30 min. Then, the substrate was rinsed, incubated in 2 % 3-triethoxysilylpropan-1-amine (APTES) solution and in poly(acrylic) acid (PAA) subsequently after the protocol described in ???. Then a 150 μm microfluidic channel was glued to the functionalized substrate and eventually filled with 1 mg mL^{-1} of neutravidin or streptavidin-atto488, if fluorescent labeling was intended. A constant flow rate of 80 $\mu\text{L min}^{-1}$ was selected for the bead capture assays under the light microscope. The general process chain is depicted in ??.

As a final result, a chemical and biological functionalization could be established to a similar degree as unspecific binding. Measuring the count of beads per occupied area yields Fig. 17a. Again, two distinct states can be observed for biotinylated and plain beads at every modified surface.

The density of bound protein could also be varied through the incubation concentration and measured reliably both in a optical bead capture assay and the MFI of the channel in a fluorescence microscope.(Figs. 17b and 17c) The relative MFI indicates here the fraction supra the non-functionalized, PBS-filled microchannel.

However, a substantially different long-term stability could neither be measured nor compared to similar results accurately. Also, no proof for the covalent functionalization could be brought with the available methods. Nevertheless, a robust and working protocol has been developed after significant literature values which encourages the position.

1.5.3. Affinity-based Concentration Measurements in the Magnetic Flow Cytometer

In this section, the results of beads rolling a neutravidin-covered channel surface will be discussed. In order to measure the interaction properties diligently, beads were functionalized with biotin at several saturation degrees ranging from 0 % to 100 % total

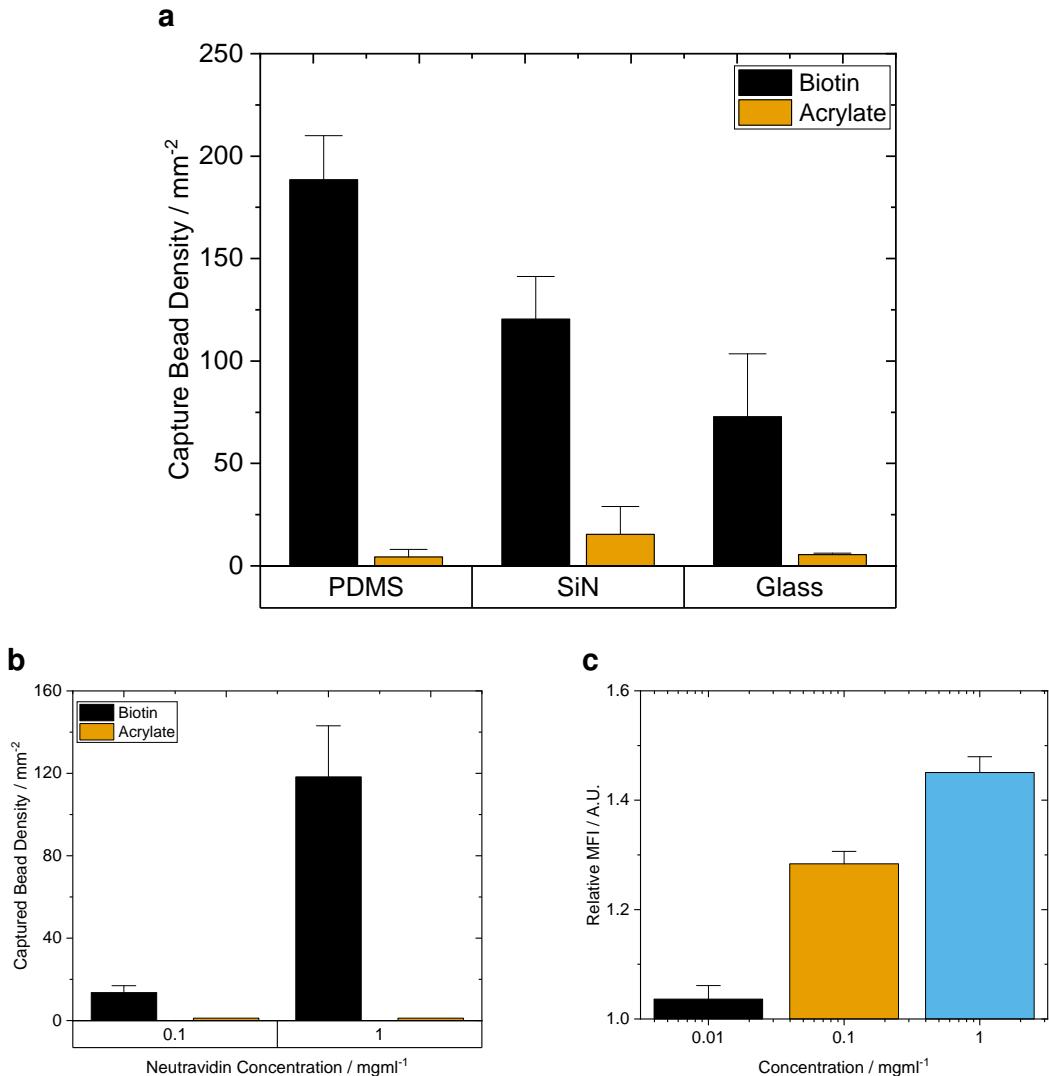


Figure 17: Optical Bead Capture Assay and Titration Fluorescence Analysis

(a) Density measurement of captured beads on the covalently functionalized surfaces of PDMS, Si_3N_4 and glass. PDMS shows the most promising density. (b) Titration of neutravidin on covalently functionalized glass with a subsequent analysis by a bead capture assay. (c) Titration of streptavidin-atto488 on covalently functionalized glass with subsequent fluorescence microscope imaging. The scale indicated the fraction above the fluorescence signal of a blank microchannel.

coverage of the available surface charges. Measurements for absolute counts as well as their time stability were carried out at $80 \mu\text{L min}^{-1}$ in a microchannel with cross-section $700 \mu\text{m} \times 150 \mu\text{m}$ (w x h). Fig. 18a shows the absolute, linearly corrected counts determined by the magnetic flow cytometer, with a defined particle concentration of $7.8 \mu\text{L}^{-1}$ to $10 \mu\text{L}^{-1}$. Clearly, a significant yes-no answer can be established by fully- vs. non-functionalized beads. Also, this measurement demonstrates high reproducibility with three different chip setups and measurement days. For approximately $\frac{2}{3}$ biotinylated beads, a gradation in counts can also be observed. However, due to the instability of the channel surface over the course of many sequential measurements, additional experiments have to be conducted to reinforce these results.

An evaluation of the time-domain distribution of the counted beads shows a very stable

baseline for fully biotinylated beads with a recovery in the single digit range per unit volume. In the negative control of unbiotinylated beads, also a noisy mean count around 100 % can be observed.⁵

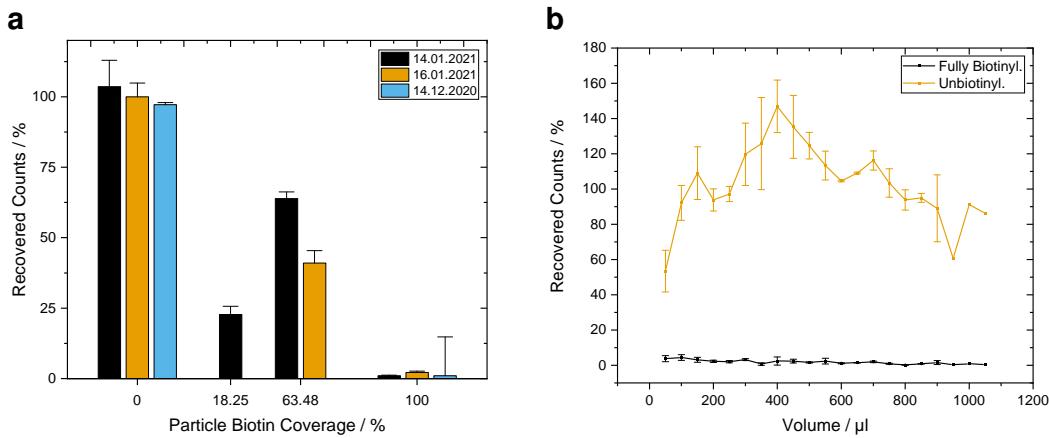


Figure 18: Reproducibility of Concentration Measurements with Saturated Neutravidin Surfaces

Biological replicates of concentration measurements with the magnetic flow cytometer. The channel surface was modified with 1 mg mL^{-1} neutravidin over night. (a) Mean and SEM of C_{const} adjusted counts for various biotinylation degrees. (b) Time series of the mean from the three measurements on the left. Biotin-beads are captured completely, thus very few counts are detected. (—) The unbiotinylated sample shows the initial ascent due to the in Sec. 1.4.1 discussed error which is related to the filling of the magnetophoretic structures. (—)

something about velocity?

⁵ The shown data corresponds to the mean of three measurements from Fig. 18a