Rotational actuation of magnetic nanoparticle clusters for solution-based biosensing

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Rotational actuation of magnetic nanoparticle clusters for solution-based biosensing

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Eindhoven, op gezag van de rector magnificus, prof.dr.ir. C.J. van Duijn, voor een commissie aangewezen door het College voor Promoties in het openbaar te verdedigen op dinsdag 6 maart 2012 om 16.00 uur

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Rotational actuation of magnetic nanoparticle clusters for solution-based biosensing

In-vitro diagnostics deals with the quantification of specific molecules or cells in a sample outside a living organism in order to highlight a particular physiological or pathological state. Samples are most easily taken from body fluids such as blood, saliva or urine. Detectable molecules with diagnostic value are called biomarkers. Examples of protein biomarkers are cardiac troponin (cTn) for the diagnosis of heart infarction or prostate specific antigen (PSA) for prostate cancer. Commonly antibody coated micro- or nano-particles are used to capture and extract biomarker molecules from a sample, because antibodies enable specific capture and because particles have a high surface to volume ratio. Magnetic particles have as additional advantage that they can be reliably manipulated by magnetic fields because biological materials are hardly magnetic. As a consequence magnetic particles are widely applied in in-vitro diagnostic assays.

The detection of biomarkers in biological samples is complex due to the low concentrations of the biomarkers and the complicated nature of the sample matrix. Biosensing relies on specific interactions, but non-specific interactions can easily occur with abundantly available endogenous molecules. Traditionally, biological assays use series of different process steps in order to achieve good detection sensitivity and specificity. One of the aims of this dissertation is to explore the feasibility of a one-step technology based on the rotation of clusters of magnetic nanoparticles and to demonstrate rapid and sensitive detection of protein biomarkers directly in blood plasma.

The first chapter of this dissertation provides an overview of the research project and describes the state of art in the field of magnetic cluster
assays, which are based on biomarker-induced binding between magnetic particles. Our literature search highlights the lack of a background-free detection principle for magnetic cluster assays. This thesis presents a novel background-free detection principle based on frequency-controlled rotation.

The rotational dynamics of magnetic particle clusters shows a critical frequency. The critical frequency is the maximum frequency at which the clusters can rotate synchronously with the external field. We model the rotational dynamics of individual two-particle clusters by deriving the equations of motion in an external rotating field (chapter 3). The theoretical model is based on the microscopic properties of the particles, in particular the magnetic susceptibility, a permanent magnetic moment of each cluster, and the size of the particles. We demonstrate that the rotational dynamics is most reproducible when the magnetic interaction is dominated by interactions between induced magnetic dipoles.

In chapter 4 we describe how frequency-controlled rotation and optical scattering give a background-free detection principle. Clusters of magnetic particles have a uniaxial symmetry, in contrast to single particles which are essentially spherical. Rotating clusters expose an angle-dependent cross-section to an incoming light beam and thereby modulate the intensity of scattered light. Single particles hardly rotate and hardly generate a modulation of light. We demonstrate that the frequency-dependence of the optical modulation signal accurately reveals the number of clusters in solution and the value of the critical frequency. The method can be applied to an ensemble of nanoparticles and allows a measurement of cluster size and the magnetic susceptibility.

The population of clusters can be modified by the addition of cluster-inducing biological molecules. We have studied molecule-induced nanoparticle binding using biotinylated Bovine Serum Albumin (bBSA) and streptavidin-coated nanoparticles, in buffer and in human plasma. Detection is performed by magnetic rotation and optical scattering, with an optical probing volume of approximately 1 nL. Using a two-step assay with a total assay time of less than 3 minutes, we demonstrate dose-response curves with a detection limit of 0.4 pM bBSA in buffer and 5 pM bBSA in human plasma.

To achieve a one-step homogeneous assay format, the non-specific interactions need to be investigated and reduced (chapter 5). We analyze the effect of pulsed rotating magnetic fields on the amount of formed clusters, both specifically and non-specifically. We identify an optimum in the time between pulses and relate it to the diffusive properties of the nanoparticles. We hypothesize that the field-on period generates a concentration of particles, while during the field-off period the particles randomize their orientations which enhances the biomarker-induced binding, and the particles have a lower non-specific binding due to a reduced contact time. To further reduce the non-specific interactions, we modify the surface molecular architecture of the
nanoparticles by means of a double layer of polymer linkers. Antibodies are linked to the particles by a primary layer giving good molecular mobility. Using the antibody as anchoring point, we attach a secondary layer of linkers in order to further shield the particles without hindering the possibility for specific binding.

We demonstrate a one-step homogeneous PSA immunoassay directly in human plasma, in a total assay time of less than 15 minutes. We obtain dose-response curves with detection limit of 0.5 pM PSA. We quantitatively explain the dose-response curves with a model based on discrete binding of biomarker molecules onto the nanoparticles, which allows us to independently and quantitatively extract the reaction parameters for the binding of biomarker molecules onto the nanoparticles and for the biomarker-induced binding between nanoparticles.

When performing an assay over several orders of magnitude in biomarker concentration, clusters of more than two particles appear. Chapter 6 presents an extension to the theoretical model presented in Chapter 3 and its experimental verification for clusters of different sizes.

In conclusion, we have demonstrated a one-step homogeneous immunoassay by means of a new and versatile bionanotechnology based on rotating magnetic nanoparticles, which will enable a wide range of further studies in optics, magnetics, biophysics and biosensing.
SAMENVATTING

Rotationele actuatie van clusters van magnetische nanodeeltjes voor biomoleculaire detectie


De detectie van biomarkers in biologische monsters is complex vanwege de lage concentratie van de biomarkers en vanwege de complexe samenstelling van de monstervloeistoffen. De detectie van biomarkers wordt gedreven door de specifieke interacties, maar niet-specifieke interacties spelen een verstorende rol vanwege de overvloedig aanwezig zijnde endogene moleculen. Traditioneel maken biologische analyses gebruik van series van verschillende processtappen om een goede detectiegevoeligheid en specificiteit te bereiken. Een van de doelen van dit proefschrift is te onderzoeken of een één-staps technologie haalbaar is gebaseerd op de rotatie van clusters van
magnetische nanodeeltjes, om snelle en gevoelige detectie van eiwitbiomarkers in bloedplasma mogelijk te maken.

Het eerste hoofdstuk van dit proefschrift geeft een overzicht van het onderzoeksproject en beschrijft de laatste technieken in het gebied van assays gebaseerd op magnetische deeltjes die tot clusters gebonden zijn via biomarkers. Onze literatuurstudie laat zien dat er nog geen principe is voor de detectie van magnetische clusters zonder een achtergrondsinaal van enkele deeltjes. Dit proefschrift beschrijft een nieuw detectieprincipe gebaseerd op frequentiegecontroleerde rotatie.

De rotatie van clusters van magnetische deeltjes laat een zogeheten kritische frequentie zien. De kritische frequentie is de maximale frequentie waarop een cluster synchroon met het externe veld kan draaien. We modelleren de rotatie van individuele twee-deeltjes clusters door de bewegingsvergelijkingen in een extern roterend veld af te leiden (hoofdstuk 3). Het theoretisch model is gebaseerd op de microscopische eigenschappen van de deeltjes, met name de magnetische susceptibiliteit, een permanent magnetisch moment van ieder cluster en de afmetingen van de deeltjes. We tonen aan dat de rotatiebeweging het meest reproduceerbaar is wanneer de magnetische interactie gedomineerd wordt door interactie tussen geïnduceerde magnetische dipolen.

In hoofdstuk 4 beschrijven we hoe frequentiegecontroleerde rotatie en optische verstrooiing leiden tot een achtergrondvrij detectieprincipe. Clusters van magnetische deeltjes hebben een uniaxiale symmetrie, in tegenstelling tot enkele deeltjes die in essentie een bolvormig zijn. Roterende clusters bezitten een hoekafhankelijke doorsnede ten opzichte van een inkomende lichtbundel en moduleren daardoor de intensiteit van het verstrooide licht. Enkele deeltjes roteren vrijwel niet en generen ook vrijwel geen modulatie. We tonen aan dat de frequentieafhankelijkheid van het optische modulatiesignaal nauwkeurig het aantal clusters in de oplossing en de waarde van de kritische frequentie laat zien. De methode kan worden toegepast op een ensemble van nanodeeltjes en voorziet in een manier om clustergrootte en magnetische susceptibiliteit te bepalen.

De populatie van clusters kan aangepast worden door de toevoeging van biologische moleculen welke clustervorming veroorzaken. We hebben molecular geïnduceerde bindingen tussen nandeeltjes onderzocht met behulp van gebiointyneerde Bovine Serum Albumine (bBSA) en nanodeeltjes gecoat met streptavidine, in buffer en in menselijk plasma. Detectie is uitgevoerd met magnetische rotatie en optische verstrooiing, met een optisch meetvolume van ongeveer 1 nL. Met behulp van een twee-staps methode met een totale analysetijd van minder dan 3 minuten tonen we dosis-respons curves met een detectielimiet van 0.4 pM bBSA in buffer en 5 pM bBSA in menselijk plasma.

Om een een-staps homogeen analyseprincipe te bereiken moeten de niet-specifieke interacties onderzocht en verminderd worden (hoofdstuk 5).
We analyseren het effect van gepulseerde roterende magnetische velden op het aantal gevormde clusters, zowel specifiek als niet specifiek. We identificeren een optimum in de tijd tussen pulsen en relateren het aan de diffusie-eigenschappen van de nanodeeltjes. We veronderstellen dat in de periode waarin het veld aan staat de deeltjes geconcentreerd worden, terwijl wanneer het magneetveld uit staat de deeltjes zich willekeurig verspreiden, wat de biomarkergeïnduceerde binding verbetert, terwijl de deeltjes minder niet-spezifieke interacties hebben vanwege de verkorte contacttijd. Om de niet-spezifieke interacties nog verder te reduceren voorzien we de nanodeeltjes van een moleculaire oppervlaktestructuur bestaande uit een dubbele laag polymere linkers. Antilichamen worden verbonden aan de deeltjes door een primaire laag die een goede mobiliteit geeft. Met de antilichamen als bevestigingspunt brengen we een tweede laag van linkers aan, om de deeltjes af te schermen zonder de mogelijkheid tot het vormen van specifieke binding te belemmeren.

We laten een een-steps homogene PSA immunoassay zien direct in menselijk plasma, met een totale analysetijd van minder dan 15 minuten. We verkrijgen dosis-respons curves met een detectielimiet van 0.5 pM PSA. We verklaren kwantitatief de dosis-respons curves met een model gebaseerd op discrete bindingen van biomarker moleculen aan de nanodeeltjes, wat ons in staat stelt om onafhankelijk en kwantitatief de reactieparameters voor de binding van biomarker moleculen op het nanodeeltje en voor de biomarkergeïnduceerde verbindingen tussen nanodeeltjes te vinden.

Wanneer een analyse wordt uitgevoerd over verschillende ordes van grootte van biomarkerconcentraties, worden clusters gevonden van meer dan twee deeltjes. Hoofdstuk 6 beschrijft een uitbreiding van het theoretische model dat in hoofdstuk 3 is getoond, samen met de experimentele verificatie voor clusters van verschillende afmetingen.

Samenvattend hebben we een één-steps homogene immunoassay door middel van een nieuwe en veelzijdige bionanotechnologie aangetoond, gebaseerd op roterende magnetische nanodeeltjes, welke een breed scala aan verdere studies in optica, magnetisme, biofysica en biosensing mogelijk zal maken.


CHAPTER 1

Introduction
1.1 – Immunoassays

In-vitro diagnostics encompasses a wide range of medical devices, with the aim to provide reliable and accurate diagnosis by testing physiological or pathological states outside a living organism. The diagnosis of many diseases can be assisted by detecting a target molecule, for example a protein such as prostate specific antigen (PSA) or cardiac troponin (cTn). Detection involves capturing target molecules and distinguishing them from other substances in a body fluid sample. Selective capturing can be done by using antibodies. Antibodies are proteins produced by the immune system with a three dimensional structure that binds to the target protein (also called antigen) with high affinity (see chapter 2). The high selectivity and specificity of the bond between an antibody and an antigen allows capturing and tagging targets and forms the basis of immunoassays. Currently most immunoassays are carried out in centralized laboratories with equipment that is operated by dedicated laboratory personnel. Inside the immunoassay, the antibody-antigen is coupled to a label, for example an enzyme or a particle, to facilitate detection in complex matrices (see Fig. 1.1). High sensitive detection is often achieved by adding steps like dilution, washing and chemical or biochemical amplifications. Technological advances are enabling the miniaturization and integration of devices for application in decentralized laboratories (see Fig. 1.2). Decentralized testing is important for time-critical diagnoses, where time wasted affects the health or even the chance of survival of the patient. The trend towards decentralization poses demanding constraints on assay technologies. An important challenge is that target molecules are available in body fluids in minute concentrations. To achieve high sensitive detection, the biochemistry of the sensing device, the so-called molecular biosensor, needs to be very efficient and well controlled to transduce the presence of specific targets into a measurable signal. Assay performance can be significantly

Fig. 1.1) Configurations for a sandwich immunoassay. Two antibodies, binding on different sites of the target, form the sandwich. One antibody is attached to a solid phase (surface in panel a and a magnetic particle in panel b) and the other is used as label. Panel c sketches the principle of a magnetic cluster assay, object of this dissertation. Both carrier solid phase and label are magnetic particles.
enhanced by the use of antibody coated particles. The high surface-to-volume ratio of the particles enables rapid and efficient capture and moreover, the labeling of targets.

1.2 – Superparamagnetic nanoparticles

Magnetic nanoparticles have the advantage that they can be externally manipulated by means of magnetic fields because biological materials are hardly magnetic. Magnetic particles consist of a core of magnetic material (iron, nickel cobalt...) often embedded in or coated with a polymeric matrix to ease biological functionalisation. Nanometer-sized particles usually contain a single magnetic grain, whereas multiple grains can be present when the diameter of the particles is larger than a few tens of nanometers. Particularly interesting is the class of superparamagnetic nanoparticles, which consists of thousands of nanometer-sized grains of magnetic material. Each grain exhibits a magnetic moment which fluctuates in orientation thanks to the thermal energy. In absence of an applied magnetic field, the sum of all the magnetic moments leads to negligible macroscopic magnetization at room temperature. The main advantage is that the aggregation of the particles is reversible and triggered by the presence or absence of an external magnetic field.

Magnetic particles historically found their primary application in magnetic separation of cells or molecules. The bioactive nanoparticles are incubated with the sample until the desired molecular or cellular species are captured onto the particle surface. Thereafter, by means of magnetic fields the particles are attracted to a surface and the supernatant is removed, allowing for
the extraction of the specific molecular or cellular species. The species can be eluted and dispersed in a clean and pure matrix. This general principle has been applied to a variety of applications: cell counting\(^5\), detection of parasites in blood\(^6, 7\) and DNA extraction prior to amplification\(^8\). Magnetic particles are also used as carriers for enzyme linked immunosorbent assays (ELISAs)\(^9\). ELISA tests, developed in the 1970s, use immobilization onto a solid surface. The amount of antigen in the sample is quantified by binding to a specific antibody that is tagged with an enzyme. The excess of tagged antibodies is removed by a washing step. A chemical compound is added, the enzyme converts it and induces a chromatic change or luminescence that can be quantitatively related to the concentration of antigen in the sample. Magnetic particles can be used as solid surface, thus achieving concentration and purification through magnetic separation, achieving faster and more sensitive assays\(^10\).

The most recent applications employ nanoparticles as labels for surface-based biosensing. Targets bind onto a sensor surface where a first type of antibodies is immobilized. Nanoparticles decorated with a second type of antibody complete the sandwich immunoassay, thus tagging the targets. Detection of the concentration of the target is performed by quantifying the number of bound nanoparticles. One particle detection technique is based on the giant magnetoresistance (GMR) effect\(^11\), whose application in biosensors leverages existing sensing technology utilized in hard disk drives. GMR is a phenomenon wherein a change in the local magnetic field induces a change in resistance due to spin-dependent scattering in magnetic multilayer or films. The magnetic nature of the nanoparticles is then exploited for detection of the
stray field they generate\textsuperscript{12-14}. Alternatively, the nanoparticles are detected optically\textsuperscript{15}, for example by exploiting their absorption and scattering properties\textsuperscript{16}. In all cases, the ability to manipulate the magnetic particles by magnetic field gradients is the essential property that serves a variety of applications, ranging from separation and transport to enabling more rapid and efficient assays\textsuperscript{17,18}.

1.3 –Magnetic cluster assays

Cluster assays (also called agglutination or aggregation assays) were introduced in the 1950s\textsuperscript{19}. The assay principle is that target molecules trigger clustering of bioactive particles, so that the number and size of the clusters is proportional to the concentration of the target. Consequently, the detection is achieved by correlating the degree of clustering in solution to the concentration of the target. Table 1.1 presents an overview on the topic of cluster assays, with particular emphasis on magnetic cluster assays. The first demonstrated detection technologies were based on biphasic response, that is, clustering causes precipitation which is observed by the naked eye. Subsequently, more quantitative detection was achieved by optical turbidimetry or nephelometry. Later, oxygen channeling chemiluminescence\textsuperscript{20} has been introduced as multi-step cluster assay, achieving femtomolar limit of detection in diluted serum. An important drawback of non-magnetic cluster assays is the slow kinetics of the aggregation process\textsuperscript{21}.

Magnetic cluster assays employ magnetic nanoparticles as labels. They consist of the sequence of an incubation phase, an aggregation phase and detection of the formed clusters. The target is immobilized on the particle.

Fig. 1.4) Possible detection principles for the magnetic nanoparticles in a surface-based biosensor. The left panel shows detection of double stranded DNA by means of GMR sensors\textsuperscript{13}. The right panel portraits an example of optical detection, by means of frustrated total internal reflection microscopy\textsuperscript{16}.
Table 1.1) Summary of particle cluster assays, listed in terms of detection technique, limit of detection and total assay time. For non magnetic cluster assays, given the extensive amount of published literature, only the original paper that introduced the technique and an example of recent development are given.

<table>
<thead>
<tr>
<th>Cluster Assay</th>
<th>Detection Technique</th>
<th>Target</th>
<th>Performances</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non magnetic</strong></td>
<td>Biphasic response(^\text{19}) (1956)</td>
<td>Gamma Globulin</td>
<td>LoD: NA Time &gt;2 hours</td>
</tr>
<tr>
<td></td>
<td>Chemiluminescence(^\text{20}) (1996)</td>
<td>TSH HBsAg</td>
<td>LoD: 10 fM, diluted serum LoD: 1 pM, diluted serum Time &lt; 25 min</td>
</tr>
<tr>
<td><strong>Magnetic</strong></td>
<td>Turbidimetry(^\text{21}) ESPCI (2006)</td>
<td>Ovalbumin</td>
<td>LoD: 1 pM in buffer Time &lt; 5 min</td>
</tr>
<tr>
<td></td>
<td>NMR (T(_2)) (^\text{22, 23}) Harvard Univ. (2007)</td>
<td>hCG Avidin</td>
<td>LoD: 1 nM in buffer Time &gt;1 hour</td>
</tr>
<tr>
<td></td>
<td>Cluster Diffusivity(^\text{24}) EPFL (2009)</td>
<td>bBSA Avidin</td>
<td>LoD: 3 pM in buffer Time &lt; 20 min</td>
</tr>
<tr>
<td></td>
<td>Modulated Transmission(^\text{30-33}) Tokyo Univ. (2010)</td>
<td>Avidin</td>
<td>LoD: 100 pM in buffer Time &lt; 1 min</td>
</tr>
<tr>
<td></td>
<td>Modulated Optical Scattering(^\text{26, 27}) Philips &amp; Eindhoven Univ. (2011)</td>
<td>bBSA PSA</td>
<td>LoD: 5 pM in plasma LoD: 500 fM in plasma Time &lt; 15 min</td>
</tr>
</tbody>
</table>

surface during the incubation phase. In a sandwich immunoassay, the nanoparticles bind to different sites of the target, enabling cluster formation for example by means of polyclonal antibodies or monoclonal antibodies which target different epitopes. The detection can either occur optically or magnetically.

An overview of literature on magnetic cluster assays is presented in Table 1. Baudry and co-authors at ESPCI demonstrated that by applying a uniform magnetic field the nanoparticles align into uniaxial chains and, because of the confined arrangement, they interact extremely rapidly\(^\text{21}\). The interpretation given is that, because of their superparamagnetic nature, the particles, even if aligned in chains, are free to rotate and find the correct
orientation for the binding to occur. The detection is performed optically and in the bulk volume of the sample: the relative decrease in the transmitted light (before and after clustering occurred) is related to the amount of clustering in solution. The increment in the speed of the aggregation reduces the total assay time from a few hours to a few minutes.

Magnetic detection by means of Nuclear Magnetic Resonance is pursued by Harvard University and T2 Biosystems. They detect the onset of clustering by monitoring the shift in relaxation time of the protons of water in biological samples. At EPFL a chip technology has been investigated with a cloud of nanoparticles transversally manipulated across a microfluidic channel. The dynamic cloud captures the target molecules. Thereafter magnetic confinement is used to focus all the nanoparticles on the side of the channel and after removal of the field the diffusion process is monitored by video-microscopy. The diffusive expansion is inversely proportional to the degree of aggregation.

Park et al. at Tokyo University introduced rotation of long chains of magnetic particles combined with optical detection. Magnetic fields are applied for a long time to allow the chains to reach an equilibrium length through magnetic aggregation. The transmitted light is dependent on the angular orientation of the chains and it is consequently time-dependent. The addition of target molecules generates an increase in the average length of the chains, which induces an increase in the modulation of the transmitted light, which is related to the concentration of targets.

All the aforementioned techniques measure changes of a large background signal resulting from particles present in the bulk volume of the sample. The large background signal is particularly critical when dealing with minute concentration of target: the sample is characterized by only a very low number of clusters in the midst of a large abundance of single nanoparticles. To achieve sensitive detection it is crucial to develop a detection technique that does not generate a large background signal, to be able to probe the prime stages of colloidal aggregation. In this dissertation, we demonstrate that rotational actuation can deliver a powerful solution to this challenge.

1.4 – Research goal and thesis outline

The aim of this dissertation is to investigate a novel nanobiotechnology to perform biomarker detection with magnetic nanoparticles in
Fig. 1.5) Schematic of the evolution of the Ph.D. project. Individual doublets have been studied by video-microscopy (panel a), to characterize their rotational behavior in the bulk of the fluid. The study led to a novel technique for magnetic characterization of the nanoparticles. Ensemble opto-magnetic detection has been achieved by frequency-selective rotation (panel b). The rotating clusters exhibit a time-dependent cross-section to the incoming light, thus modulating the intensity of the scattered light. The modulation is proportional to the number and size of clusters in solution. Panel c) shows the protocol for our homogeneous immunoassay based on frequency-controlled rotation. Rapid cluster formation is achieved by pulsating the magnetic field. The field is removed prior to detection to allow for re-dispersion. A weak rotating field is applied for detecting the presence of clustering in the sample. Panel d) shows two assays we studied. Biotinylated BSA has been detected by a two-step model assay and PSA in a one-step immunoassay. A double layer of polymer linkers generates a robust surface architecture against non-specific interactions in blood plasma (panel c).

The bulk volume of complex matrixes. In particular we study the use of magnetic fields to manipulate and cluster magnetic particles and to demonstrate the feasibility of a one-step homogeneous cluster immunoassay.
The research project can be divided in three main phases. Initially, rotational magnetic actuation was chosen as potential actuation protocol and the rotational dynamics of individual two-particle clusters was characterized (Fig. 1.5a). Subsequently, an opto-magnetic platform to detect an ensemble of rotating clusters was designed and assembled (Fig 1.5b). Its most distinctive feature lies in being only sensitive to rotating clusters of nanoparticles, resulting in a background-free detection principle. An actuation protocol based on repeated magnetic pulses achieves rapid cluster formation by enhancing randomization of the relative angular orientation between neighbor nanoparticles (Fig 1.5c). Non-specific interactions are a limiting factor when measuring in complex biological matrices. Accordingly, the final part of this dissertation will describe how rotation, combined with a molecularly engineered surface architecture is able to reduce non-specific clustering in complex matrices (Fig. 4d).

Chapter 2 gives an overview of the theoretical background of this thesis and a broader outlook on the state of art. Chapter 3 deals with the study of the rotational behavior of single two-particle nano-clusters. The rotational motion is studied by means of video-microscopy and a theoretical model based on the microscopic properties of the particles allows for accurate magnetic characterization and estimation of the local viscosity of the fluid medium. The influence of the rotation in bulk or in close proximity to a surface is addressed. The regime where dipole-dipole interaction is the dominant source of magnetic torque is identified as the regime that allows for more reproducible torque exertion on the uniaxial clusters by optical scattering. Chapter 4 describes how to perform opto-magnetic detection of ensembles of rotating uniaxial nano-clusters. By extending the use of magnetically-driven rotation to the detection phase, we achieve an innovative and background-free technology for sensitive detection of the first stages of colloidal aggregation. The analysis in the frequency domain of the optical signals relates to the size and number of rotating nano-clusters and allows for ensemble magnetic characterization. To characterize the performances of the opto-magnetic platform, a two-steps model assay in human plasma is presented. Chapter 5 describes the implementation of detection of a cancer biomarker (Prostate Specific Antigen) in blood plasma. By combining pulsation of the nanoparticles during the phase of cluster formation and engineering the surface molecular architecture, we demonstrated a rapid and highly-sensitive one-step immunoassay in untreated
complex matrix. Chapter 6 extends the theoretical framework to longer chains of chemically-bound nanoparticles.
References

1. http://cobas.com/Products/Pages/cobas6000analyzerseries.aspx
Chapter 2

Theoretical background
2.1 – Introduction

As described in the previous chapter, the goal of this research project is to develop a novel nanotechnology to detect protein biomarkers in blood plasma. Our approach is based on rotation of superparamagnetic nanoparticles, detected by means of their scattering properties. Accordingly, in this chapter we provide an overview of the theoretical tools on which this dissertation builds upon by answering to few questions. What is a superparamagnetic particle? What properties are important to characterize them and to understand their rotational dynamic? How does light interact with magnetic nanoparticles? How are the target biomolecules captured by the antibodies on the nanoparticles? How are the antibodies immobilized onto the nanoparticle surface?

The following sections address briefly the aforementioned topics. Section 2.2 reports an overview of magnetism providing a basic understanding of the origin of different classes of magnetic materials and of the superparamagnetic behavior of a nanoparticle. Section 2.3 reports on the experimental techniques to characterize the magnetic properties of superparamagnetic nanoparticles and the experimental results relative to the nanoparticles used in the experimental section. The exertion of rotation and torque to magnetic nanoparticles (and relative applications to biological systems) is object of Section 2.4. In section 2.5 we give a brief introduction to the phenomenon of optical scattering by means of particles whose physical dimensions are comparable to the wavelength. When performing an immunoassay, target biomolecules need to be captured by the antibodies, phenomenon which can be described by means of Langmuir kinetics, reported in section 2.6. The last section of this chapter describes the molecular structure of antibodies and the origin of the specificity and selectivity of the binding with an antigen. Basic bioconjugation techniques to couple the antibodies onto the nanoparticles and onto polymer linkers are also object of section 2.7.

2.2 – Magnetism

The laws of electromagnetism are elegantly described by Maxwell’s equations, which show that magnetic fields are generated by the motion of charged particles, that is, electric currents. Since the atomic structure of any material contains electrons orbiting around the nucleus (current loops), they are expected to be influenced by an external magnetic field. The result of such
interaction is that in presence of external fields, matter exhibits a magnetization \( M \) (defined as the magnetic moment per unit volume) which is related to the external field \( H \) by the magnetic susceptibility \( \chi \): 

\[
\vec{M} = \chi \vec{H}
\]  

(2.1)

Depending on the value of \( \chi \), a material can be diamagnetic \((\chi < 0)\), paramagnetic \((\chi > 0)\) or ferromagnetic \((\chi \gg 0)\). Ferromagnetic materials exhibit the strongest response when exposed to a magnetic field. The physical origin lies in the exchange interaction: because electrons are indistinguishable, Pauli’s exclusion principle dictates that electrons with parallel spins must differ in the spatial part of the wave function, that is, they cannot occupy the same orbital. Consequently, the wave function with parallel spins has lower energy than the antiparallel configuration, partially filled orbitals will present themselves with parallel spins and therefore crystalline materials (made of one ferromagnetic element) will have a macroscopic magnetization.

The nanoparticles used in the experimental section are referred to as superparamagnetic. As the name suggests, superparamagnetic particles exhibit magnetic properties only in presence of an externally applied field and their response is stronger than the one of a paramagnetic material. Such behavior is due to their microscopic structure: a myriad of nanometer-sized grains of iron oxides (mainly magnetite, \( \text{Fe}_3\text{O}_4 \), with possibly some maghemite, \( \text{Fe}_2\text{O}_3 \)) embedded in a polymer matrix (polystyrene). Some oxides (i.e. magnetite) exhibit ferrimagnetic properties, that is, their structure is characterized by two sublattices of different ions, exhibiting magnetic moments of different magnitude oriented in opposite directions. As a result, a net magnetization is present in the bulk material.

The bulk of ferromagnetic and ferrimagnetic materials is divided into zones of uniform magnetization, called Weiss domains. Domains can maintain uniform magnetization up to a certain critical size (of the order of tens of nanometers for magnetite) and different domains are separated by Bloch walls, inside which the magnetization gradually rotates. Balancing the total dipolar energy and the domain wall energy, it can be deduced that below a certain critical size of the whole ferrimagnetic object, no magnetic domains will be formed. This size may be roughly estimated by the typical thickness of a domain wall, which is, in case of magnetite\(^2\), approximately 80 nm. This is significantly larger than the typical bead grain size of 6 to 12 nm and therefore the magnetization of a single grain may be assumed uniform in one direction\(^3\).
The preferred orientation, so-called easy axis, of the magnetization of each domain depends on several types of magnetic anisotropy.

Magnetocrystalline anisotropy is the result of the interplay between the geometry of the crystal structure and the spin-orbit coupling. Essentially, the nucleus of an atom induces a magnetic field in the reference frame of each electron, which is parallel to its orbital angular momentum. However, the electron carries a magnetic moment itself; the coupling between the two influences the actual direction of the spin. Consequently, the actual position of the nuclei influences the direction of the spins of the electrons, giving origin to a preferred orientation of the magnetization.

Shape anisotropy is related to the shape of the magnetic domain: the magnetic poles generate a field inside the material, which tends to demagnetize the material and thus minimize the potential energy. Depending on the shape, the demagnetizing field has components of different magnitude, resulting in a preferred direction for the magnetization. Spherical objects do not exhibit shape anisotropy since the demagnetizing field is identical regardless of the direction. In practice however, the grains are unlikely to be perfect spheres and it is possible to demonstrate that minute deviations from spherical symmetry translate in such a strong interaction that shape anisotropy completely dominates over magnetocrystalline anisotropy.

Another source for anisotropy are the spins at the surface of the grain, which experience only half of the neighboring spins as compared to the spins in bulk magnetite, i.e. surface anisotropy. The spins at the interface will tend to either align to the bulk magnetization or lie in the same plane as the grain surface. In this way the surface anisotropy roughly reduces the strength of the shape anisotropy by a factor that may be estimated by exploiting the ratio between the interface volume and the bulk volume of the grains. Note that the interface has a thickness which is partially determined by the surface roughness. A precise estimation of the importance of surface anisotropy over shape anisotropy is cumbersome, given the many uncertainties in exact shape and roughness of the grains. In the following, it will be assumed that shape anisotropy is dominant over surface anisotropy: the reported values for surface anisotropy are comparable to shape anisotropies of an object with aspect ratio comprised between 1.2 and 2.

We assume that an individual grain is a single domain with uniaxial anisotropy and it can relax its magnetization to be parallel or anti-parallel to one single axis. To flip the direction of magnetization, the thermal energy ($k_B T$)
needs to be large enough to overcome the anisotropy barrier, which is
determined by the domain size. The time constant descriptive of such process
is given by:

$$\tau = \tau_0 e^{KV/kB\tau}$$  \hspace{1cm} (2.2)

where K is the (shape) anisotropy constant, V is the volume of the domain, and
\(\tau_0\) relates to the Larmor frequency. From equation (2.2) it is possible to see that
a critical volume exists below which the magnetization flips orientation
extremely rapidly. When \(\tau\) is below few seconds, the material is said to exhibit
superparamagnetic behavior.

A superparamagnetic particle is characterized by thousands of these
single domain particles, therefore the net magnetization is averaged to zero, in
contrast to the bulk material. When an individual nanoparticle is in presence of
an external field, the magnetic moments of each grain align and the energy of
interaction is given by \(\mu B \cos \theta\), with \(\theta\) the angle between easy axis and field.
When an ensemble of nanoparticles is considered, fluctuations due to thermal
energy result in a distribution of orientations of the magnetization. Such a
process can be statistically described by the canonical ensemble, consequently
the probability that a magnetic moment assumes a given orientation with
respect to the field is given by a Boltzmann factor:

$$p(\theta) \propto e^{\frac{\mu B \cos \theta}{k_B\tau}}$$  \hspace{1cm} (2.3)

By noticing that the number of moments oriented in an infinitesimal angle
\(\theta + d\theta\) is proportional to the fractional surface area of a surrounding sphere,
the normalized integral over all possible orientations yields the angular
distribution of the moments. Each moment contributes a factor \(\mu \cos \theta\) to the
magnetization, consequently the macroscopic magnetization is:

$$M = N\mu(\cos \theta) = N\mu \int_0^\pi \cos \theta \ p(\theta) \ d\theta$$  \hspace{1cm} (2.4)

where N is the total number of moments. This leads to the Langevin equation,
descriptive of the magnetization of the ensemble:

$$M = M_{sat} \left[ \cot(\alpha) - \frac{1}{a} \right], \quad \text{with} \quad \alpha = \frac{\mu B}{k_B\tau} \quad \text{and} \quad M_{sat} = N\mu \quad (2.5)$$
In equation (2.5) $\mu$ is the magnetic moment of the superparamagnetic particle, $M_{\text{sat}}$ is the saturation magnetization and $B$ is the magnetic field. In the limit of small values of $\alpha$, that is, for small values of applied field and for very high temperatures, equation (2.5) can be expanded at first order, which yields a linear relationship between magnetization and applied field. The constant of proportionality is the (paramagnetic) susceptibility, in consistency to equation (2.1).

2.3 – Magnetic characterization of nanoparticles

The aim of this section is to provide a magnetic characterization of the nanoparticles used in the experimental section. The relevant parameters are the saturation magnetization ($M_{\text{sat}}$) and the magnetic susceptibility ($\chi$). Due to the processes of synthesis, the number and size of the grains varies between different nanoparticles. Moreover, the nanoparticles are also characterized by a size distribution, resulting in variability in the values of particle susceptibility.

Several techniques exist for measuring the saturation magnetization and the magnetic susceptibility of nanoparticles, where the most renowned are SQUID magnetometers and Vibrating Sample Magnetometer (VSM). In both techniques, the magnetic flux through a loop is altered by the presence of the magnetic nanoparticles and the change in current is related to the magnetic properties of the sample.

In time varying fields, the nonzero relaxation time of the grains can cause the induced magnetization to have a time delay with respect to the field. The time delay can be accounted for by a complex frequency dependent magnetic susceptibility:

$$\chi(\omega) = \chi'(\omega) - i\chi''(\omega)$$  (2.6)

Fig. 2.1) VSM measurements of Ademtech 500 nm (left) and Dynal 1 $\mu$m (right). Measurements done by Kim van Ommering.
The magnetic moment of each grain is able to align with the external field extremely rapidly; however when the external excitation acts on a time scale comparable to the relaxation time of the grains each magnetic moment cannot completely align with the field, affecting the value of the susceptibility. Dynamic measurements can be performed on ensemble of magnetic nanoparticles by sweeping the frequency of the external field and by registering the change in the flux. Typically the complex part of the susceptibility becomes significant only for frequencies above hundreds of Hz.

VSM measurements have been performed to characterize the nanoparticles used in the experiments (see Fig. 2.1). MyOne nanoparticles from Dynal are found to be characterized by a saturation magnetization $M_{\text{sat}}=0.01$ A/m and a nanoparticle susceptibility of $\chi=1.5\times10^{-18}$ m$^3$. Ademtech 500 nm are found to be characterized by a saturation magnetization $M_{\text{sat}}=0.004$ A/m and a nanoparticle susceptibility of $1.7\times10^{-19}$ m$^3$. By calculating the slope in the linear part of the magnetization curve, it is possible to estimate the value of the magnetic susceptibility, which is respectively given by $\chi_{1\mu\text{m}} = 2.7$ and $\chi_{500\text{nm}} = 2.6$.

The nanoparticles also retain a small permanent moment which is a locked magnetization that does not vanish at zero applied field. Possibly, larger grains above the superparamagnetic limit, which require more than the thermal energy to randomize their orientation, are responsible for the presence of a permanent moment. The discovery of the presence of a permanent moment is recent and a standard technique to accurately determine it is lacking. VSM measurements did not highlight the presence of a permanent moment. In chapter 3 and 4, we demonstrate that rotation can provide information about the values of the permanent and induced moments of individual nanoparticles and of ensembles of nanoparticles. In the following section, an overview of the state-of-art of the experiments on magnetic torque and rotation is given.

2.4 – Rotation and torque

Rotation of superparamagnetic particles has been investigated by different approaches (see table 1). In this section, an overview of the published literature on the topic is given. A novel class of single particles have been investigated by Michigan University. A chemical process for synthesizing so called MagMOONs, micrometer sized nanoparticles half-coated with metal to ease the visualization
of the rotation under a microscope\textsuperscript{17}, has been developed. The MagMOONs are used as physiochemical sensors and to follow the kinetic of binding of single bacteria to the rotating nanoparticles\textsuperscript{15}.

Single superparamagnetic nanoparticles have also been used to apply torque to nucleic acids at Delft University\textsuperscript{18, 19}. DNA molecules are rotated and supercoiling is achieved. A similar approach is followed at Sorbonne University, where DNA is studied by torsional manipulation and the data are interpreted in terms of structural rearrangements of the nucleosome\textsuperscript{20}. Torsional manipulation by means of single particles is applied to protein complexes at Eindhoven University\textsuperscript{14, 21}. The torque is quantitatively related to the permanent moment of individual nanoparticles and the stiffness of the complex is studied as function of torque strength and number of bonds per nanoparticle.

Long chains are used for biosensing in combination with fluorescent labeling of the target molecule at Arizona University\textsuperscript{22}. Long chains are rotated and the fluorescent signal is monitored as function of the target concentration. They achieve a limit of detection in the nanomolar range. Manipulation of long chains is exploited by several research institutions to achieve effective mixing in microfluidic chambers\textsuperscript{23-25, 30}. Such studies focus on

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<thead>
<tr>
<th>Rotation of magnetic particles</th>
<th>Approach</th>
<th>Research Institution</th>
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<tbody>
<tr>
<td>Single</td>
<td>Biosensing using fluorescent labels</td>
<td>Michigan Univ. \textsuperscript{15}</td>
</tr>
<tr>
<td></td>
<td>Torsion of nucleic acids</td>
<td>Delft Univ.\textsuperscript{18, 19} Sorbonne Univ.\textsuperscript{20}</td>
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<tr>
<td></td>
<td>Torsion of proteins</td>
<td>Eindhoven Univ.\textsuperscript{21}</td>
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<tr>
<td>Clusters</td>
<td>Biosensing using fluorescent labels</td>
<td>Arizona Univ.\textsuperscript{22}</td>
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<td></td>
<td>Mixing</td>
<td>Eindhoven Univ.\textsuperscript{23} Madrid Univ.\textsuperscript{24, 29} Seul National Univ.\textsuperscript{25}</td>
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<td>Biosensing using long chains</td>
<td>Tokyo Univ.\textsuperscript{26}</td>
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<td></td>
<td>Biosensing using small clusters</td>
<td>Philips &amp; Eindhoven Univ.\textsuperscript{27, 28}</td>
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Table 2.1) Summary of the state-of-art in torque and rotation, using single magnetic particles and clusters.
chains of magnetically-clustered nanoparticles, and the length of the chains is usually determined by the magneto-rheology and the hydrodynamic conditions. A similar approach is followed at Tokyo University for biosensing. By adding a target biomolecule to the sample the length of the chains increases; thus modulating the optical transmittivity of the sample. The modulation is correlated to the target concentration, achieving a limit of detection of the order of hundreds of picomolar.

In this dissertation we present the equations of motion of two-particle clusters and an extension for chains of N nanoparticles. Such theoretical background allows us to identify the regime of reproducible torque, which we exploit for developing an opto-magnetic platform for biosensing.

2.5 – Optical scattering

In this dissertation, rotating clusters of nanoparticles are detected by means of their optical response. In particular, the light scattered by the particles is detected in dark field and related to the number and size of clusters (see chapters 4 and 5).

When electromagnetic radiation encounters matter the radiation couples to the electronic cloud in the particle, which results in an induced electric dipole. The oscillating induced dipole moment is manifested as a source of electromagnetic radiation, thereby resulting in scattered light. The majority of light scattered by the particle is emitted at the identical wavelength of the incident light, a process referred to as elastic scattering. Different scattering regimes exist depending on the size parameter, ratio between wavelength and physical dimension of the scattering particles. If the scattering particles have a characteristic size much smaller than the wavelength, the scattering is described by the so-called Rayleigh regime. When the particle has physical dimensions comparable with the wavelength, an analytical solution of Maxwell’s equations for the scattering is provided by Mie theory. By expressing the incident and scattered radiation as superposition of opportune basis functions and exploiting the linearity of Maxwell’s equations, the solution of the problem translates to finding opportune values for the matrix of the expansions coefficients and numerous efforts have been done to develop methods for computing them.

The detailed scattering profile is dependent on the microscopic structure of the particles, medium and incident radiation. A few general statements can be made. Primarily, when radiation hits one (or more) particles,
the flux is decreased, resulting in extinction of the incident beam. Such decrease is not only due to scattering, but also derives from absorption, non-radiating decay of the excitation and conversion into thermal energy. In fact, every object can be described by a complex refractive index, whose real part accounts for the phase of the propagating wave, while the imaginary parts describe the absorption of radiation by the object. It can then be demonstrated that the rate of extinct energy is proportional to the incident intensity:

\[ W_{\text{ext}} \propto \sigma_{\text{ext}} \cdot I \] (2.7)

where the constant of proportionality represents the extinction cross section, given by the sum of the absorption and scattering cross section:

\[ \sigma_{\text{ext}} = \sigma_s + \sigma_a \] (2.8)

The cross section, as defined in (2.7) and (2.8), refers to the light scattered over a full solid angle. In our experimental configuration, the scattered light is collected by a lens with limited numerical aperture, therefore collecting only light scattered over a limited surface. It is still possible to relate the intensity of the scattered light to the incident radiation by means of the so-called differential cross section. The integral of the differential cross section over a full solid angle yields the scattering cross section, as defined in (2.8). Moreover, the angular distribution of the scattered light is dependent on the particular value of the size parameter, therefore the angular pattern of the scattered light is wavelength dependent for particles of fixed size.
By measuring the properties of the incident and scattered radiation, many properties of the scattering medium can be inferred. The first phenomena that could be explained by Mie scattering belong to atmospheric science. An extensive amount of literature describes the scattering properties of particles from individual spheres, arbitrary shaped particles and colloids, however there is still lack of consensus when non-idealities, such as surface roughness and intrusions, need to be taken into account.

2.6 – Langmuir kinetics

The capture of biomarkers onto the nanoparticles is a time-dependent process. A theoretical description is given by the Langmuir model, originally developed to quantify the adsorption of gas on a solid phase as a function of concentration and temperature. The model is based on the assumptions that the surface is homogeneous, covered with equivalent binding sites that can host only one target. It is also assumed that depletion of targets is negligible and adjacent targets do not interact.

The model describes the equilibrium state of the following process:

\[ A + S \rightleftharpoons AS \]  
(2.9)

where \( A \) is the concentration of the adsorbing species (targets) and \( S \) is the concentration of adsorption sites on the solid phase. By imposing that the total amount of targets is the sum of free and adsorbed targets, the adsorption kinetics is described by the following equation:

\[
\frac{d\theta(t)}{dt} = C \cdot k_{on}[1 - \theta(t)] - k_{off}\theta(t)
\]  
(2.10)

Fig. 2.3) Schematic representation of an adsorption/desorption mechanism on a (flat) solid phase. The binding sites \( S \) are represented by antibodies whereas the target \( A \) is drawn as red crosses. The absorption (desorption) process occurs with a rate proportional to \( k_{on} \) (\( k_{off} \)).
where \( \theta(t) \) is the fraction of bound targets, \( C \) is the concentration of available targets and \( k_{on} \) and \( k_{off} \) are respectively the association and dissociation constants. The equation is valid in the regime where the concentration of targets is much larger than the concentration of capture molecules calculated over the total sample volume. Equation (2.10) gives as solution:

\[
\theta(t) = \frac{KC}{1+KC} \left[ 1 - e^{-(C \cdot k_{on} + k_{off}) t} \right], \quad \text{with} \quad K = \frac{k_{on}}{k_{off}} \quad (2.11)
\]

which describes the kinetics of binding. The saturation value of \( \Gamma(t) = KC/1 + KC \) is descriptive of the fraction of targets that are bound on the solid phase at equilibrium (Langmuir adsorption isotherm). The time constant \( \tau = (C \cdot k_{on} + k_{off})^{-1} \) is an estimation of the time required to reach the equilibrium configuration. If the reaction is not diffusion limited, the reaction reaches equilibrium between association and dissociation in a time scale of approximately 4 or 5 times \( \tau \). For typical values of association and dissociation constant for antibody/antigen couples, \( (k_{on} \sim 10^5 M^{-1}s^{-1}, \ k_{off} \sim 10^{-4}s^{-1}) \) and clinically relevant concentrations (~ pM) the time constant is of the order of days. In our experiments, the concentrations of antibodies \( C_{Ab} \) is much larger than the target concentration, thus \( \tau = (C_{Ab}k_{on})^{-1} \).

### 2.7 – Antibodies and their bio-conjugation

Antibody coated nanoparticles are extensively used in the experimental section to bind the target to be detected. The reason for choosing antibodies to capture the target lies in the strong affinity between antibody and antigen and in the selectivity of the interaction. Affinity assays are based on the high

![Fig. 2.4) Chain representation of an immunoglobulin G. The blue and red chains represent the heavy chains while the yellow and green are the light chains](image-url)
Fig. 2.5) Schematic representation of the surface molecular architecture used to develop our immunoassays based on rotational magnetic actuation (see chapter 5).

Specificity and selectivity of the antibodies-biomarkers binding, which is due to the particular structure of the antibody. Antibodies are host proteins produced by the immune system for molecular recognition of exogenous molecules (antigen) entering the body. The antibodies circulate throughout the bloodstream and, upon binding, they allow the recognition of the antigen. Antibodies are macromolecules that belong to a particular class of proteins (glycoproteins). Proteins are polymers of linear chains of amino acids called polypeptides (molecules containing an amine group, a carboxylic acid group and a side-chain that varies between different amino acids). The structural units of the polymer, called peptides, are bound together by covalent bonds between the carboxyl group and the amine group of adjacent peptides. The details of the sequence of polypeptides define the so-called primary structure of a protein. To perform biological functions however the peptides need to arrange in a well-defined and complex three-dimensional structure. Local segments fold into a secondary structure (i.e. helixes) and the relative spatial organization of several local segments describes the so-called tertiary structure.

Antibodies are an assembly of four polypeptide chains (two identical heavy chains and two identical light chains, see Fig. 4), whose 3D arrangement defines their quaternary structure. A series of non-covalent bonds and di-sulfide bonds keeps the chains together, conferring the antibody a bilateral symmetric structure. In particular, both heavy and light chains are characterized by constant regions and variable amino terminal ends. The variable parts of heavy and light chains (paratopes) are antigen-specific and confer the antibody its specificity. Typically the immunological response to an antigen is heterogeneous and results in several cell lines. Each cell line generates
antibodies that specifically recognize the antigen in a particular site, called epitope. Antibodies purified from a family of cell lines are therefore binding to a multiplicity of epitopes and are therefore referred to as polyclonal. Individual cell lines are capable of generating one family of antibodies that can selectively bind to an individual epitope (monoclonal antibodies).

In this dissertation, antibodies are coupled with different surface chemistries on the surface of magnetic nanoparticles. Nanoparticles terminated with carboxyl groups can be directly attached to the amine groups of an antibody by EDC chemistry. EDC is a carbodiimide which reacts with carboxyl groups activating them forming an instable intermediate that binds to the amine groups on the antibodies by means of an irreversible peptide bond.

The amine groups are localized on different positions of the antibody. Consequently, the anchoring point is highly ill-defined, resulting in paratopes non-oriented with respect to the surface of the nanoparticles. Only a fraction of the antibodies is therefore sterically accessible for binding the antigen. Our aim is however to both orient the antibodies as well as minimizing the non-specific interactions, so that non-specific clustering doesn’t hinder the performance of our biosensor.

We achieve this goal by mediating the coupling of antibodies on nanoparticles by linker molecules. The advantage with respect to the EDC surface chemistry is that the antibodies may present themselves with a mild outwards orientation, resulting in a higher fraction of active and sterically accessible antibodies. The method chosen in this dissertation for coupling antibodies with linkers is by means of biotin/avidin reaction (see Fig 2.5). The highly specific interaction of avidin with biotin is the strongest known non-covalent interaction of a protein and ligand, which guarantees strong binding to the nanoparticles. Moreover, several biotinylated linker molecules are commercially available with terminal ends (i.e. NHS-esters) which can react with the amine group of the antibodies, enabling ease of crosslinking. With such an approach, we have developed a modular surface architecture (see chapter 5): biotinylated antibodies and linkers are bound on the surface of the nanoparticles, forming a primary layer. A second layer of linkers is bound to the first, to improve the suppression of non-specific interactions in blood plasma.
References

Chapter 3

Magnetically-controlled rotation and torque of individual two-particle clusters

Abstract. We demonstrate the controlled rotation and torque generated by uniaxial magnetic clusters formed by two bound superparamagnetic particles in a fluid. The torque and rotation are precisely controlled by rotating magnetic fields, generated by an external electromagnet or by on-chip current wires. We present the magnetic energy equations and the equations of motion for two-particle clusters, with contributions from the permanent and induced magnetic moments of the particles. A comparison of theory and experiments allows an estimation of the different moments with accuracy better than 10% across a wide frequency range. At low frequencies and low magnitudes of the applied magnetic field, both the permanent and induced moments of the particles have contributions to the torque. At either high fields or high frequencies, the torque is dominated by the induced moment. The predictability of the torque is highest in the regime of low frequencies and high field, where the torque has a large magnitude and is determined by the magnetic shape anisotropy of the cluster. A comparison of rotation in bulk fluid and on a chip surface shows an increase of friction by a factor 9 originating from the surface proximity. The detailed understanding of the torque and rotation of two-particle uniaxial magnetic clusters opens a range of possibilities in lab-on-a-chip applications, such as the actuation of single molecules, fluid mixing in microfluidic chambers, and novel cluster-based assays.

This chapter (except section 3.5) is based on Ranzoni et al., Lab on a Chip, 2010, 10 (2), 179-188.
3.1 - Introduction

The controlled movement of micro and nano-actuators in fluid is of high interest for lab-on-a-chip technology. Particularly interesting are actuators that can be controlled by magnetic fields because they can be precisely applied and they do not perturb biological fluids. The most well-known types of magnetic actuators are micro- and nanoparticles. Biological molecules and cells can be attached to magnetic particles, for transportation, separation, and detection\textsuperscript{1-6}. Furthermore magnetic actuators can be used to mix and transport fluids\textsuperscript{7,8}. One of the new dimensions in actuation is the application of rotation and torque. Magnetic particles can be rotated in order to coil biological molecules and study their properties\textsuperscript{9-13}, fluids can be rotationally actuated by rotating ensembles of unbound magnetic particles\textsuperscript{14}, and rotating single particles can be used to probe fluid mechanical properties\textsuperscript{15}. Although rotational actuation has been applied, the control of torque by magnetic clusters is still in its infancy. Janssen et al. demonstrated that a controlled torque can be generated by a single spherical superparamagnetic particle\textsuperscript{16}. The torque is quantified by the micromagnetic structure of the particles, in particular the presence of a small permanent magnetic moment. Unfortunately the use of single spherical particles to exert torque in lab-on-a-chip applications has three drawbacks: (i) the permanent magnetic moment was shown to vary considerably from particle to particle\textsuperscript{16}, (ii) the orientation of the permanent moment in the particle is not coupled to an external physical axis, and (iii) the precise rotation of spherical particles is relatively hard to detect. Therefore a novel cluster entity is needed, having quantifiable rotation and torque properties and a well-defined response to an external magnetic field.

In this paper we describe the rotation and torque of clusters consisting of two chemically bound spherical micro- or nano-particles (see Figure 3.1). The uniaxial symmetry of the cluster enables accurate detection of angular orientation and rotation, while a well-controlled torque is generated by the uniaxial magnetic shape anisotropy caused by the magnetic dipole–dipole interaction between the two particles. The aim of this study is to provide a validated theoretical model to support the use of two-particle clusters in lab-on-a-chip applications, for a wide range of applied field frequencies. The description of the clusters is based on three magnetic interactions: the interactions between the field and the induced moments of the particles, between the field and the permanent moments of the particles, and the
interaction between the two induced moments. A fit of the theoretical model to the measured time-dependent rotation of the clusters yields values for the magnetic susceptibility and the permanent moment of the particles. From our experiments we derive the magnitude and precision of torque that can be applied by two-particle clusters, and we apply the clusters to estimate the ratio of their rotational viscous drag in bulk fluid and in fluid near a chip surface.

3.2 - Theory

We consider the case of a cluster of two superparamagnetic particles floating in a viscous liquid in the presence of an external magnetic field $\vec{H}$, rotating in the $xy$ plane with angular velocity $\omega_f$. Each particle contains...
nanometer-sized single-domain grains of ferrimagnetic material embedded in a polymer matrix\textsuperscript{17}. The total magnetic moment of the particle is given by the vector sum of the individual magnetizations of the grains. To first order in the field, the induced magnetic moment $\vec{m}_i$ of the particle is given by $\vec{m}_i = \chi V \vec{H}$\textsuperscript{18}, with $\chi$ the susceptibility of the particle material and $V$ the volume of the particle. This linear approximation is valid for $H \ll H_{sat}$, where $H_{sat}$ is the field that saturates the magnetic moment of particle. In time varying fields, the non-zero relaxation time of the grains can cause the induced magnetization to have a time delay with respect to the field. The time delay can be accounted for by a complex frequency-dependent magnetic susceptibility\textsuperscript{18}:

$$\chi(\omega_f) = \chi'(\omega_f) - i\chi''(\omega_f)$$  \hspace{1cm} (3.1)

Typically the complex part of the susceptibility becomes significant only for frequencies above hundreds of Hz\textsuperscript{19}. In addition to a frequency-dependent induced moment, the particles can have a permanent magnetic moment originating from a small fraction of large grains ($\geq 20$ nm) that can be present inside the superparamagnetic particle\textsuperscript{16,20}. In the following, we will refer to low field strength when the magnetic moment of the particle is dominated by the permanent moment, and to a high field strength when the induced moment is dominating.

In the following we will derive the equations of motions for a two-particle cluster in an applied rotating magnetic field. The equations are based on the magnetic properties of the particles (the induced and permanent magnetic moments of each particle and the magnetic coupling between the particles) and the viscous properties of the fluid medium. We will derive the equations by calculating the magnetic torque generated by the two-particle cluster in the applied rotating field. An alternative theoretical approach based on the Lagrangian formalism is given in the Appendix.

First we calculate the magnetic energy, which allows us to calculate the magnetic torques regardless of the frequency range. In a dipole approximation, the magnetic energy of a two-particle cluster in the external field is the sum of three contributions, namely of the field interacting with both individual dipoles and the two dipoles interacting with each other:

$$U = -\vec{m}_1 \cdot \mu_0 \vec{H} - \vec{m}_2 \cdot \mu_0 \vec{H} + \frac{\mu_0}{4\pi (2R)^3} [\vec{m}_1 \cdot \vec{m}_2 - 3(\vec{m}_1 \cdot \vec{d})(\vec{m}_2 \cdot \vec{d})]$$ \hspace{1cm} (3.2)
where $\vec{m}_1$ and $\vec{m}_2$ represent the total magnetic moment of particle 1 and 2, consisting of the sum of their permanent and induced magnetic moments, where $\hat{d}$ is a unitary vector connecting the centers of the two particles, $\mu_0$ is the permeability of the vacuum, $R$ is the radius of an individual particle and $2R$ is the distance between the two dipole centers. (3.2) can be split into two terms, namely $U_i$ containing energy terms of the induced moments ($\vec{m}_i$) and $U_p$ containing energy terms of the permanent moments of the particles ($\vec{m}_p$):

$$U_i = -m_{i1}\mu_0 H\cos(\varphi_f - \varphi_{i1}) - m_{i2}\mu_0 H\cos(\varphi_f - \varphi_{i2}) +$$
\[+ \frac{\mu_0}{4\pi} \frac{m_{i1}m_{i2}}{(2R)^3} \cos(\varphi_{i1} - \varphi_{i2}) - 3\cos(\varphi_{i1} - \varphi_c)\cos(\varphi_{i2} - \varphi_c)\]  \hspace{1cm} (3.3)

$$U_p = -m_{p1}\mu_0 H\cos(\varphi_f - \varphi_{p1}) - m_{p2}\mu_0 H\cos(\varphi_f - \varphi_{p2}) +$$
\[+ \frac{\mu_0}{4\pi} \frac{m_{p1}m_{p2}}{(2R)^3} \cos(\varphi_{p1} - \varphi_{p2}) - 3\cos(\varphi_{p1} - \varphi_c)\cos(\varphi_{p2} - \varphi_c)\]  \hspace{1cm} (3.4)

where we have assumed that all vectors lie in the $xy$ plane of rotation of the applied magnetic field. In (3.3) and (3.4) $m_{i1}$, $m_{i2}$ are the induced moments of particles 1 and 2, $m_{p1}$, $m_{p2}$ the permanent moments of particle 1 and 2, and $\varphi_0$, $\varphi_{i1}$, $\varphi_{i2}$, $\varphi_{p1}$, $\varphi_{p2}$, $\varphi_c$ are respectively the orientations of the applied field, the induced moments, the permanent moments and the axis of uniaxial symmetry of the cluster with respect to the $x$ axis. We can simplify (3.3) by assuming that the particles have equal susceptibility and that the individual induced magnetic moments are equal and parallel to each other ($\varphi_{i1} = \varphi_{i2} = \varphi_i$). Under these assumptions $U_i$ becomes:

$$U_i = -\frac{8}{3} \pi R^3 \chi \mu_0 H^2 \cos(\varphi_f - \varphi_i) + \frac{\pi \mu_0 H^2 \chi^2 R^3}{18} [1 - 3\cos^2(\varphi_i - \varphi_c)]$$  \hspace{1cm} (3.5)

The configuration of minimum energy corresponds to the situation in which the cluster and the induced moments are aligned with the field ($\varphi_{ma} = \varphi_{i1} = \varphi_{i2} = \varphi_f$). A deviation from this state increases the magnetic energy $U_i$ and generates a torque on the cluster via the induced moments:

$$\tau_i = \frac{\partial U_i}{\partial (\varphi_{i1} - \varphi_c)} + \frac{\partial U_i}{\partial (\varphi_f - \varphi_i)} =$$
\[\frac{\pi}{6} R^3 \chi^2 \mu_0 H^2 \sin[2(\varphi_i - \varphi_c)] + \frac{8}{3} \pi R^3 \chi \mu_0 H^2 \sin(\varphi_f - \varphi_i)\]  \hspace{1cm} (3.6)
We can also derive the torque generated by the permanent moments. The total permanent magnetic moment of the two-particle cluster is given by $\mathbf{m}_p = \mathbf{m}_{p1} + \mathbf{m}_{p2}$. Due to the small magnitude of the individual permanent moments, the term describing the interaction between the two moments in (3.4) can be neglected, giving: $U_p = -m_p \mu_0 H \cos(\varphi_f - \varphi_p)$ and thus a torque:

$$\tau_p = \frac{\partial U_p}{\partial (\varphi_f - \varphi_p)} = m_p \mu_0 H \sin(\varphi_f - \varphi_i)$$

(3.7)

The orientation of the permanent moment of the cluster is fixed with respect to the axis of cylindrical symmetry; in the following we will then assume that $\varphi_p = \varphi_c - \vartheta$, with $\vartheta$ the angle between the permanent moment and the cluster axis. We note that (3.6) and (3.7) are consistent with the definition of magnetic torque $\mathbf{\tau} = \mathbf{m} \times \mu_0 \mathbf{H}$.

The clusters experience a viscous drag that counteracts the magnetic torque. In microfluidics experiments the Reynolds number is generally much lower than one, so we will neglect terms of fluid inertia. The hydrodynamic torque of a single sphere rotating around a non-central axis has been derived by Happel and Brenner. The rotation of a two-particle cluster around the $z$-axis passing through the centre of mass corresponds to two spheres rotating around a non-central axis (Figure 3.1). As a starting point, we approximate the total viscous torque on the two-particle cluster by the sum of the torques exerted onto two particles rotating in isolation, neglecting the weak coupling between the particles. With the rotational frequency of the cluster $\omega_c = d\varphi_c / dt$, and $\eta$ the viscosity of the fluid medium, the hydrodynamic torque is given by:

$$\hat{\tau}_{\text{hydro}} = -2\pi \eta \eta_{\text{eff}} R^3 \omega_c$$

(3.8)

with the effective viscosity $\eta_{\text{eff}} = c \eta$. For $c = 1$, the cluster experiences the same viscosity as two isolated spheres that rotate around a single axis in a bulk fluid without any hydrodynamic coupling with the walls. The constant $c$ can be used to account for hydrodynamic coupling, between the two particles (weak coupling) or between the particles and a nearby wall (strong coupling).

One way to describe the interplay between viscous and magnetic effects is by a dimensionless parameter, namely the Mason number, defined as the ratio between rotational shear forces and magnetic interaction forces. In literature, the influence of a permanent moment has been neglected and the magnetic forces are usually considered to be proportional to the square of the
induced magnetization\(^{11,23}\). As a consequence, such a definition remains valid as long as \(\chi VH \gg m_p\). A more general expression, valid regardless of the strength of the magnetic field, should include also the presence of a permanent moment. We therefore propose the following definition for the Mason number \(Mn = \tau_{\text{hydro}}/(\tau_i + \tau_p)\).

We will first focus on the low frequency regime, where \(\chi' \gg \chi''\). The direction of the induced moment in each particle is given by the local field, which slightly deviates from the orientation of the external field due to the particle–particle interaction, \(i.e.,\) due to the shape anisotropy of the two-particle cluster. The condition of minimum energy of the two-particle cluster occurs when the axis of cylindrical symmetry is aligned with the external field \(\varphi_f = \varphi_c\). When a phase lag is present (\(i.e., \varphi_f \neq \varphi_c\)) the system is not in global equilibrium\(^{14}\) and \(\partial U_i / \partial \varphi_c \neq 0\). Nevertheless, since the period of the external field is much longer than the relaxation time of the grains, the induced magnetic moments can be considered at every instant in time in equilibrium with the field, \(i.e.,\) the quantity \(U_i\) is in a local magnetic energy minimum with respect to \(\varphi_i\). The corresponding condition \(\partial U_i / \partial \varphi_c = 0\) can be exploited to calculate the behavior in time of the magnetic moment:

\[
\sin(\varphi_i - \varphi_f) + \frac{\chi'}{16}[2(\varphi_i - \varphi_c)]
\]

Using (3.9) and typical values of \(\chi'\) between 1 and 3, we find that the maximum value of \(\varphi_i - \varphi_f\) is between 3° and 10°. Equation (3.9) depends both on \(\varphi_{ma}\) and \(\varphi_i\) and can be solved when coupled with the equation of motion that balances the magnetic and hydrodynamic torques. With \(\varphi_f = \omega_f t\), evaluating \(\tilde{\tau}_i + \tilde{\tau}_p + \tilde{\tau}_{\text{hydro}} = 0\), and taking the time derivative of (3.10), we obtain a set of coupled differential equations:

\[
\frac{d\varphi_c}{dt} = \frac{\mu_0 H^2(\chi')^2}{168\eta_{\text{eff}}} \sin[2(\varphi_i - \varphi_c)] + \frac{m_p \mu_0 H}{14\pi \eta_{\text{eff}} R^3} \sin(\omega_f t - \varphi_c + \vartheta) \quad (3.10)
\]

\[
\frac{d\varphi_i}{dt} = \frac{\omega \cos(\varphi_i - \omega_f t) + (\chi'/8) \cos[2(\varphi_i - \varphi_c)] \frac{d\varphi_c}{dt}}{\cos(\varphi_i - \omega_f t) + (\chi'/8) \cos[2(\varphi_i - \varphi_c)]} \quad (3.11)
\]

The parameter \(\chi''\) is absent in (3.9), (3.10) and (3.11) due to the low-frequency approximation. The right hand side of (3.10) is the sum of two sinusoidal functions, which can be positive as well as negative. In fact, a maximum frequency of synchronous rotation exists, beyond which the cluster
exhibits periodic backward rotations. The maximum angular velocity at which the cluster is able to rotate synchronously with the field is called the critical frequency.

Just above the critical frequency, the cluster rotates with a superimposed wiggling motion, because periodically the phase lag between the field and the cluster orientation \( \alpha = \omega_f t - \varphi_c \) is larger than 90°. The result is that the net rotation rate of the cluster is lower than the field and decreases as a function of the field frequency. The wiggle frequency depends on the mechanism generating the magnetic torque. If the torque due to the permanent moment is significant, then the wiggle period equals the time required for the field to make a complete rotation around the cluster \([\omega_w = \omega_f - \omega_{net}]\). If the torque is only generated by the induced moment, then the period of a wiggle equals the time required for the field to make a half-turn around the cluster \([\omega_w = 2(\omega_f - \omega_{net})]\) because the induced moment has the freedom to flip 180° along the uniaxial symmetry axis of the cluster.

Now we will estimate the magnitude of the critical frequency \( \omega_{crit} \). Since the direction of magnetization of the individual particles is very close to the actual direction of the applied magnetic field, we shall assume that \( \varphi_i = \varphi_f = \omega_f t \). Taking the first derivative of (3.9) with respect to the phase lag \((\alpha = \omega_f t - \varphi_c)\), we find the configuration that maximizes the magnetic torques:

\[
2 \omega_{crit}^i \cos(2\alpha) + \omega_{crit}^p \cos(\alpha + \vartheta) = 0
\]

where

\[
\omega_{crit}^i = \frac{\mu_0 H^2(\chi_t)^2}{16\pi \eta_{eff}}; \quad \omega_{crit}^p = \frac{m_p \mu_0 H}{14\pi \eta_{eff} R^3}
\]

For low fields, when the permanent moment is larger than the induced moment, the permanent moment has a significant influence on the cluster behavior. When the permanent moment of the cluster is aligned with the cluster axis, it is possible to obtain an analytical expression for the critical frequency:

\[
\omega_{crit} = \frac{1}{2\pi} \left(2 \omega_{crit}^i x + \omega_{crit}^p \right) \sqrt{1 - x^2}
\]

where \( x = \left[ -\omega_{crit}^p + \sqrt{(\omega_{crit}^p)^2 + 32(\omega_{crit}^i)^2} \right] / (8 \omega_{crit}^i) \) and the critical phase lag is given by \( \alpha_{crit} = \cos^{-1}(x) \). When the induced moment is much larger than the
permanent moment, the critical frequency equals $\omega_{\text{crit}}^i$ and scales quadratically with the strength of the applied magnetic field. In this regime it is possible to derive an analytical expression that relates the critical frequency to the wiggling frequency and the net frequency of rotation. In the approximations that $\varphi_i \approx \varphi_f$ and $m_p = 0$, the equation of motion in terms of the phase lag $\alpha$ becomes:

$$\frac{d\alpha}{dt} = \omega_f - \omega_{\text{crit}}^i \sin(2\alpha) \quad (3.15)$$

An analytical expression for the period of a wiggle can be derived by integration from $-\pi/2$ to $\pi/2$, which gives:

$$\omega_w^2 = 4(\omega_f^2 - \omega_{\text{crit}}^2) \quad (3.16)$$

which relates the critical frequency to the field frequency and the wiggling frequency. This expression is of interest because it allows one to estimate the critical frequency from a single wiggling frequency determination at a given, fixed frequency of the rotating field. The value of the critical frequency can then be used to determinate the induced magnetic moments and the torque applied.

We will now focus on the high-frequency regime, wherein the rotation frequency of the field is much higher than the rotation period of the cluster. In this regime the time-averaged contributions to the torque of the permanent moment and of the dipole–dipole interaction are zero. The torque generated by the non-zero relaxation time of the magnetic grains inside the particle, reflected in a non-zero imaginary part of the susceptibility $\chi''$, becomes dominant. The relaxation time causes an angle between the field and the induced magnetic moment of the particles, giving rise to a torque according to (3.6). Therefore the equation of motion becomes:

$$\frac{d\varphi_c}{dt} = \frac{2\mu_0 h^2}{21\eta_{\text{eff}}'} \chi'' \quad (3.17)$$

The cluster rotation rate $d\varphi_c/dt$ is proportional to the complex part of the susceptibility and is definite positive, so no wiggling behavior is expected. The rotation rate depends on the field frequency $\omega_f$ via the frequency dependency of the complex susceptibility. The maximum rotation rate occurs at the field frequency that maximizes the complex susceptibility.$^{19}$
In summary, we have defined a uniaxial cluster composed of two coupled superparamagnetic particles. We obtained a general expression for the magnetic interaction energy between the particles and the field, identifying the contributions from the induced and permanent moments, which led to a general analytical expression for the magnetic torque. Equations of motion were derived for a rotating field at low and high frequencies, balancing the magnetic and viscous torques. In the low frequency regime the induced magnetic moments are in equilibrium with the field, leading to the set of coupled differential \((3.10)\) and \((3.11)\), and to expression \((3.14)\) for the critical frequency. At frequencies much higher than the critical frequency the rotation...
rate of the cluster is proportional to the complex part of the magnetic susceptibility, see (3.17).

The mechanism of torque generation by a two-particle uniaxial cluster depends on the frequency and strength of the applied rotating magnetic field. At low frequencies and low values of magnetic field strength the torque is dominated by the permanent moment. At low frequencies and higher values of the magnetic field strength, the interaction between the induced dipoles is the driving force of the torque, being proportional to the real part of the susceptibility. At high frequencies the complex susceptibility determines the torque. In the Experimental section we will investigate which mechanism is most effective and most precise for torque generation.

3.3 - Material and methods

Magnetic clusters have been studied over a wide frequency range using two experimental arrangements, sketched in Figure 3.2. The first arrangement consists of four electromagnets driven by sinusoidal signals with 90 degrees phase shift, which creates a rotating magnetic field between the pole tips (Figure 3.2b). The voltage signals from two wave function generators (Agilent Technology 33250A) are fed into voltage-to-current converters in order to power the coils. The magnetic field was calibrated by placing the probe of a Gaussmeter between the pole tips. A PMMA vessel has been designed with a cylindrical cavity, with a diameter of 1 mm and 7 mm depth. COMSOL simulations of the magnetic field showed that the uniformity of the field is better than 5% across the 1 mm cavity diameter.

A second experimental setup was used to study cluster rotation up to high frequencies of the applied magnetic field (Figure 3.2c). The clusters are placed on a chip with embedded current wires as described in ref 16. The wires are fed with sinusoidal currents such that generate a magnetic potential well that weakly captures the cluster at the crossing point of the wires.

A microscope with high-speed camera (Allied Technology – Pike) was used to record movies at 115 frames per second. The movies were analyzed with a MATLAB script that transforms each frame of the movie in a black and white image and fits the imaged clusters with ellipsoids, in order to calculate the cluster orientations.

Uniaxial clusters were made from streptavidin-coated superparamagnetic particles (Dynabeads® MyOne™, diameter 1 μm). These
particles are highly monodisperse in size and are saturated by a field strength close to 25 mT. In order to form two-particle clusters, 2 μl of particle solution were 200 times diluted in PBS to a concentration of 50 μg ml\(^{-1}\) and mixed with biotinylated BSA at a concentration of 40 ng ml\(^{-1}\). After 30 min incubation, the binding reaction was stopped by adding 20 μl of free biotin at 2 mg ml\(^{-1}\) in order to saturate the particles with biotin. During all rotation experiments the particles were suspended in PBS buffer. The viscosity of PBS was measured by a MCR300 rheometer (Anton Paar Physica) and the value 0.001 Pa·s was found, with an accuracy of 1%. The sample was diluted such that each cluster is at least 10 diameters away from other particles, to avoid hydrodynamic or magnetic coupling between clusters. Video-microscopy was used to select two-particle clusters for experimental recordings.

3.4 - Results and discussion

The rotation of the clusters depends, according to (3.10) and (3.11), on the rotation frequency of the field \(\omega_f\), the strength of the magnetic field, the value of the magnetic susceptibility and of the permanent moment, and on the effective viscosity \(\eta_{eff}\). We have recorded the motion of individual two-particle clusters in a frequency scan, which was repeated for different strengths of the magnetic field. Figure 3.3 shows rotation data for a two-particle cluster at a field strength of 1.17 mT. Panel (a) shows a sequence of recorded images. The net rotation rate of the cluster is plotted as a function of frequency in panel (b). At low frequencies the cluster rotates synchronously with the field. Beyond the critical frequency (at about 9 Hz) the cluster exhibits a rotation with wiggling motion and the net rotation rate decreases strongly as a function of frequency. The data in panel (b) and (c) have been fitted to (3.11), yielding a particle susceptibility \(\chi = 2.40 \pm 0.06\) and a permanent moment \(m_p = (1.25 \pm 0.10) \times 10^{-16}\) A·m\(^2\), which leads to a critical frequency \(\omega_{crit} = 9.4\) Hz. Using these values and (3.5), (3.6) and (3.7), we find that the magnetic energy and the torque are between two and three orders of magnitude larger than the thermal energy \(k_BT\). Therefore the torque applied to the cluster is hardly influenced by Brownian fluctuations. Above the critical frequency, the measured data show a wiggling frequency that is equal to the difference between the field frequency and the net rotation frequency of the cluster: \(\omega_w = \omega_f - \omega_{net}\) (see panel c). This indicates that the cluster rotation is influenced by a locked permanent
Fig. 3.3) Ten movie frames of an individual cluster in bulk fluid, with a field applied by the quadrupole electromagnet. The field magnitude is 1.17 mT and the field rotation frequency is 10.5 Hz. The cluster consists of two bound particles with a diameter of 1 μm. The field frequency is higher than the critical frequency so a net rotation with wiggling motion is observed: the cluster rotates clockwise until frame (v), it stops and moves backwards in frames (vi) and (vii) until the field overtakes the cluster and it moves clockwise again. (b) The measured net rotation frequency of a single two-particle cluster as a function of the field rotation frequency. The line represents a fit of (3.10) and (3.11), with $R = 0.5 \mu m$ and $\eta_{\text{eff}} = 0.001$ Pa s (we assumed $c = 1$), with two fitting parameters: $\chi = 2.40 \pm 0.06$ and $m_p = (1.25 \pm 0.10) \times 10^{-16}$ A m$^2$. (c) Measured and calculated [using (3.10) and (3.11)] cumulative angle of rotation as a function of time for field rotation frequencies 4.5 Hz and 10.5 Hz (for clarity the 4.5 Hz curves are shifted upward). The calculation is based on the same parameters as in panel (b)

moment, as was explained in the theoretical section. The calculated rotation behavior above the critical frequency [see the 10.5 Hz curve in panel (c)] shows components at higher frequencies, which are caused by flipping of the induced moment.

To collect statistics on the particle properties, we measured the critical frequencies of a series of different two-particle clusters for several values of field strength. The results are plotted in Figure 3.4. At low fields, when the behavior is dominated by the permanent moments, we observe low critical frequencies with high variations. The variations are caused by the variability in the permanent moment and occasionally by out-of-plane rotations of the clusters$^{21}$. At high fields, the critical frequency scales with the square of the applied field and shows very low variability due to the reproducibility of the dipole–dipole interaction. The data have been fitted to (3.14) in order to
determine the average values of susceptibility and permanent moment over the ensemble of particles. We found $\chi = 2.65 \pm 0.26$ and $m_p = (1.50 \pm 0.58) \times 10^{-16}$ A·m². The same data have also been fitted to (3.13) with $m_p = 0$, which gives $\chi = 3.0 \pm 0.6$ when fitted over the whole range. Interestingly, the spread in the response of the clusters strongly reduces at higher fields and becomes as low as 4% for a field of 2 mT, in the regime when the shape anisotropy is the dominant mechanism of torque generation. In Vibrating Sample Magnetometry (VSM) measurements, we found $\chi = 2.7$ and no permanent component could be measured. Using the same 1 μm particles, Janssen et al.\textsuperscript{24} found a variation in the measured induced moments of 21%. Both the VSM values and the variation measured by Janssen are in agreement with our data.

In the theoretical section we suggested an alternative technique to measure the critical frequency of the cluster through (3.16). It has the experimental advantage of requiring only one measurement to estimate the critical frequency but, since the permanent moment was neglected, (3.16) overestimates the critical frequency. Comparing the value obtained through

![Fig. 3.4](Critical frequency as a function of applied field for several clusters in bulk fluid. For every cluster, the critical frequency was determined through the method of Figure 3.3b. Every point shows the average and the standard deviation of measurements of the critical frequency performed on seven different two-particle clusters. The relative standard deviation ($\Delta \omega_{\text{crit}} / \omega_{\text{crit}}$) strongly decreases for increasing field strength, indicating that the torque and rotation of the cluster are most accurately controlled in the regime in which the induced moment dominates the permanent moment. The lines represent fits of the data to (3.14) using $R = 0.5$ μm and $\eta_{\text{eff}} = 0.001$ Pa s. The fits allow for an estimation of the average susceptibility and permanent moment of the particles. The torque is calculated using (3.8) with $R = 0.5$ μm and $\eta_{\text{eff}} = 0.001$ Pa s. The uncertainty in the field strength was estimated to be ±2.5%; therefore the uncertainty in the value of the square of the magnetic field represented in the error bars is ±5%).
(3.16) and the measured value reported in Figure 3.4 for the highest applied field strength, we found that the precision of the single-measurement determination of the critical frequency is between 5% and 15%.

In the theoretical section we argued that a two-particle cluster should be able to rotate also at high field frequencies, when the relaxation time of the grains embedded in the polymer matrix becomes comparable to the period of the applied magnetic field. To test this hypothesis experiments across a wide

![Graph](image)

**Fig. 3.5** (a) The on-chip net rotation frequency of a two-particle cluster as a function of the applied field rotation frequency, for different magnitudes of the applied field. The lowest field strength used in the experiments is 1.7 mT, so that the rotation is dominated by the induced moment. The inset shows that critical frequency has a parabolic dependence on the applied field, in agreement with the expression for $\omega_{\text{crit}}$ in (3.13). (b) The high-frequency behavior of a single cluster for two different strengths of the magnetic field. At frequencies above approximately 1 kHz, the rotation and torque originate from the imaginary susceptibility $\chi''$. The inset shows the frequency dependence of $\chi''$ as extracted from the two curves using (3.17) with $\eta_{\text{eff}} = 8.8 \times 10^{-3}$ Pa s.
frequency range were done for a cluster suspended on a chip with embedded current wires (see Figure 3.5). Figure 3.5a shows on-chip rotation measurements using field frequencies similar to the frequencies used in the bulk experiments. Data were recorded for different magnitudes of the applied field. The presence of a critical frequency is clearly visible in the data. The critical frequency appears to be proportional to the square of the applied field [see the inset of panel (a)], which is caused by the fact that the behavior is dominated by the induced moment at the used field magnitudes.

Interestingly, it appears that the on-chip critical frequency is almost one order of magnitude lower than the values observed in the bulk fluid measurements using the quadrupole electromagnet. We attribute the difference of critical frequency to the hydrodynamic environments of the cluster: in the electromagnet setup the cluster rotates freely in a bulk fluid, while it is in close proximity to a surface in the chip setup. The presence of the surface generates a higher viscous drag on the cluster due to the zero-slip boundary condition. When the critical frequencies obtained on-chip are compared to the critical frequencies measured in bulk at the same magnetic field, a ratio
\[ \frac{\omega_{\text{crit}}^{\text{on-chip}}}{\omega_{\text{crit}}^{\text{bulk}}} = 0.114 \pm 0.002 \] is found. This means that the effective viscosity experienced by the cluster on-chip is much higher than in bulk fluid by a factor
\[ c_{\text{on-chip}} / c_{\text{bulk}} = 8.8 \pm 0.14. \]

The fact that the critical frequency is proportional to the square of the applied field (inset of Figure 3.5a) demonstrates that the factor \( c \) is independent of the field strength for the fields used in the experiment. This can be understood from the forces that the cluster experiences. In the field range between 1.7 mT and 4.3 mT, calculations show that the attractive magnetic force that pulls the cluster toward the chip surface ranges between 0.8 pN and 4.9 pN. This force is opposed by a repulsive electrostatic force generated by the equal-signed charges on the surface of the chip and on the magnetic particles. Due to the strong distance dependence of the electrostatic forces, a variation of the attractive magnetic force induces only a very small change of the actuator-to-surface distance (estimated to be less than a few nanometers) and therefore an insignificant change of the viscous drag.

On-chip experiments at much higher frequencies are shown in Figure 3.5b. The rotation frequency of the cluster is orders of magnitude lower than the frequency of the field. The cluster rotation frequency increases with field frequency until a maximum in the MHz regime. We attribute this behavior to a torque generated by the non-zero relaxation time of the grains inside the
superparamagnetic particles, which is expressed as a non-zero imaginary susceptibility $\chi''$. The behavior in the MHz regime appears to vary strongly between different clusters (not shown), which we attribute to variations in the grain size distributions in the particles. According to (3.17), measuring the average rate of rotation corresponds to measuring the complex part of the susceptibility of the particles. In the two-particle cluster measurements, the peak value of the complex susceptibility is typically found to be between 0.45 and 0.55 and the maximum is reached above 500 KHz. This value of $\chi''$ is close to values found in the literature. We found the peak of the complex susceptibility at smaller frequencies than reported in the literature, which might be due to a larger average grain size in the polymer matrix, which gives a longer relaxation time.

3.5 - Measurement of viscosity

The theoretical model we developed to describe the motion of two-particle clusters is based on the microscopic properties of the nanoparticles and of the fluid medium. In particular, the critical frequency depends on the magnetic properties of the particles (magnetic susceptibility and permanent moment) and the viscosity of the fluid.

The possibility of being able to measure the viscosity of a sample is a very interesting feature for a biosensor, especially in the context of point-of-care testing. Viscosity of blood can differ considerably between people; for example, the viscosity of blood depends on the concentration of hematocrit and it is affected by certain medications. The magnetophoretic mobility and the diffusion of the micro or nanoparticles are all dependent on the value of viscosity and consequently, each biosensor measurement may benefit from the determination of the actual viscosity of the sample.

We first determine the magnetic properties of the particles by performing the experiments in PBS buffer, whose viscosity was measured with a rheometer and does not differ appreciably from the viscosity of water. Thereafter, we proceed by studying the evolution of the ensemble critical frequency in the bulk volume of different liquid mediums, more similar to real body fluids. First, glycerol solutions of known viscosity have been prepared and the critical frequencies of 10 different two-particle clusters have been measured. The measurement has been performed in the regime where the permanent moment
can be neglected \((\mu_0H > 2\text{mT})\). Figure 3.6 shows the results of the measurement and an inverse linear relationship is found, as expected from (3.13). Thereafter, real samples of unknown viscosity have been tested with our technique. We focus on buffer (10mM PBS pH 7.4, with 5% w/v of BSA) and human plasma, since in the following chapters all the experiments aiming to quantitatively measure protein concentrations will be performed in these fluids. The results are summarized in Table 1, which highlights that the relative differences between the different fluids measured with a rheometer correlate well with our experiments.

It is beyond the scope of this dissertation to develop a technique to measure the absolute value of viscosity of a non-newtonian fluid, whose viscosity depends on the shear flow. For our scope, it is important to underline the influence of the sample viscosity on the assay parameters (see chapter 6) and that the local viscosity experienced by the clusters might vary considerably. In particular, the viscous drag experienced by the micro- and nano-clusters relates to the local concentration of other protein complexes, which can be highly inhomogeneous in the bulk volume of complex matrixes such as blood plasma. Our approach is particularly suited for biosensing since it requires submicroliter sample volumes and only a one-time calibration: the relative changes with respect to a reference sample can be exploited to adjust the magnetic actuation protocol with respect to donor-to-donor variations of viscosity.

![Fig. 3.6](image)

Fig. 3.6) Calibration curve relating viscosity and the average value of the critical frequencies for ten two-particle clusters. The critical frequency was determined by video-microscopy, averaging the measured value for \(N=10\) clusters. The viscosity of glycerol solutions can be found in Ref. 26.
<table>
<thead>
<tr>
<th></th>
<th>$\omega_{\text{crit}}$</th>
<th>$\eta_{\text{microscopy}}$</th>
<th>$\eta_{\text{rheometer}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS + 5%w/v BSA</td>
<td>3.03 ± 0.66</td>
<td>2.64 ± 0.14</td>
<td>2.2</td>
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<tr>
<td>Heparin plasma pool</td>
<td>4.50 ± 1.02</td>
<td>2.11 ± 0.19</td>
<td>1.9</td>
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<td>Single donor plasma (1)</td>
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<td>2.26 ± 0.11</td>
<td>2.3</td>
</tr>
<tr>
<td>Single donor plasma (2)</td>
<td>2.78 ± 0.59</td>
<td>2.80 ± 0.21</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Table 3.1** Comparison between viscosity values measured by monitoring the rotational behavior of individual two-particle clusters and with a rheometer.

Previous methods involved pulsated magnetic fields to orient ferromagnetic particles in the fluid\(^{27}\). Under the aid of a magnetic field, the ferromagnetic particles align and induce optical birefringence. The relaxation time of the particles in absence of the field can be then optically measured and it is dependent on the viscosity of the fluid. Since the method is based on the anisotropy of the particles, there are some restrictions in the choice of the particles and in the strength of the field, which do not apply with our approach. Moreover, in order to induce appreciable anisotropy, high density of nanoparticles is required whereas we require much lower volume fractions.

3.6 - Conclusions and outlook

We have described the use of uniaxial clusters formed by two bound superparamagnetic particles as a basic tool to exert rotation and torque in fluid. The developed theoretical model for the torque and rotation of two-particle clusters allows for characterization of the magnetic particles in terms of their permanent and induced moments at low frequencies (with precision better than 10%) and of the complex susceptibility at high frequencies. The 1 μm particles used in the experiments were characterized by an average susceptibility of 2.65 and a permanent moment of the order of $1.5 \times 10^{-16}$ A m\(^2\), with a susceptibility spread from particle to particle of 15–20%, in agreement with previous measurements. Three different regimes of torque generation have been identified, namely low-frequency and low-field strength, low-frequency and
high-field strength, and high frequency. We proved that the highest reproducibility is found in the regime of low frequency and high field, where the torque is large and generated by the magnetic shape anisotropy. A comparison of the rotation of clusters in bulk fluid and on a chip surface showed an increase of friction by a factor 8.8 caused by the surface proximity. The presented theoretical approach can be extended to clusters with more than two particles, which will generate even larger shape anisotropy.

We demonstrated that the torque of individual clusters can be accurately measured and reproducibly controlled in fluid. This opens novel possibilities in lab-on-a-chip applications with ensembles of clusters. A quantitatively controlled torque can be applied to individual biological molecules and to biological bonds with high accuracy. The use of uniaxial clusters will allow for an ensemble assay with bound particles on a surface, in which many biological bonds are simultaneously assayed to rapidly collect statistical information. In another application, the synchronous response of clusters can be exploited to effectuate fluid actuation and mixing in microfluidic chambers. Furthermore, the cluster analysis technique based on the critical frequency of rotation can be used to study fluid-dynamic interactions in geometries of different confinement and interactions at different interfaces. Finally, the principles described in this paper can be applied in so-called cluster or aggregation assays. Particles are coated with capture molecules (e.g. antibodies or oligonucleotides) that specifically bind to analyte molecules (e.g. proteins or nucleic acids). The presence of analyte in the sample causes particles to form clusters. At low analyte concentrations, the sample contains a low number of two-particle clusters against a background of a high number of non-clustered single particles, which represents a serious detection challenge. Using magnetic particles and rotating magnetic fields, we foresee the possibility to actuate and very sensitively detect the two-particle clusters and thereby the analyte molecules in the sample.
3.6 - Appendix

The equation of motion of a two-particle cluster can also be derived according to the Lagrangian formalism, which gives additional physical insight in the dynamics of rotating clusters. The system is defined as a two-particle cluster surrounded by a liquid phase which extends to infinity. As the boundary condition, the fluid is supposed to be at rest at infinity. A system of $i = 1, \ldots, N$ Lagrange equations is given by:

$$\frac{d}{dt} \left( \frac{\partial L}{\partial q_i} \right) - \frac{\partial L}{\partial q_i} + \frac{\partial F_R}{\partial q_i} = 0 \quad (A.1)$$

where $q_i$ is the $i$-th generalized coordinate and $L$ the Lagrangian of the system. The Rayleigh dissipation function $F_R$ takes account of the non-conservative forces acting on the cluster.

The Lagrangian, by definition, is given by the difference between kinetic (translational energy) and potential energy of the system. Because the Reynolds number is typically much lower than one, we can neglect inertial terms and thus the kinetic terms vanish. The Lagrangian of the system is then the opposite of the magnetic interaction energy

$$L = -(U_i + U_p) = \frac{8}{3} \pi R^3 (x' - x'') \mu_0 H^2 \cos(\varphi_i - \varphi_1) +$$

$$+ m_p \mu_0 H \cos(\varphi_i - \varphi_c + \theta) - \frac{\pi \mu_0 H^2 (x')^2 R^3}{18} [1 - 3 \cos^2 (\varphi_i - \varphi_c)] \quad (A.2)$$

Power is dissipated in the system in several ways. However we focus only on the power dissipated to overcome the viscous drag. The rationale of this choice will be addressed in the following paragraph. The power dissipated by friction of a rotating particle has been calculated by Happel and Brenner$^{22}$:

$$P_{\text{viscous}} = \vec{v} \cdot \vec{F} + \vec{\omega}_m \cdot \vec{t} \quad (A.3)$$

where $\vec{v}$ is the instantaneous velocity of the fluid, $\vec{F}$ is the hydrodynamic force exerted by the fluid on the particle by virtue of the rotational motion, $\vec{\omega}_m$ is the angular velocity of the particle and $\vec{t}$ is the torque acting on the particle. The two-particle cluster is modeled as two spheres rotating around the center of mass. In this approximation the energy dissipated by the cluster is twice the power dissipated by a single sphere spinning around that axis (see Figure 3.1):

$$P_{\text{viscous}} = 2(6 \pi \eta_{\text{eff}} R v^2 + 8 \pi \eta_{\text{eff}} R^3 \omega_c^2) \quad (A.4)$$
The Rayleigh $F_R$ function is related to the power dissipated by the liquid as $F_R = P_{viscous}/2$. At the boundary between fluid and particle $\mathbf{v} = \mathbf{\omega} \times \mathbf{R}$, so the Rayleigh dissipation function becomes:

$$F_R = 14\pi \eta_{\text{eff}} R^3 \omega_c^2$$  \hspace{1cm} (A.5)

Before proceeding we briefly comment on the generalized variables that we choose to evaluate the Lagrange equations, namely $\varphi_f - \varphi_c$ and $\varphi_i$. The first is the physical phase lag between field and cluster and therefore is suitable to derive the equation of motion of the cluster. The orientation of the induced magnetization is chosen as second variable because it is descriptive of the internal dynamics of the particles. The derived Lagrange equation is the equation of motion for the induced magnetization with a few peculiarities. The Rayleigh function is not dependent on this particular variable, which implies that this equation will describe conservative fields. Moreover, since the Lagrangian does not depend on velocity terms, the equation simplifies to $\partial L/\partial q_2 = 0$, which describes an equilibrium configuration. In the following we focus on two different cases: when the magnetic system is in equilibrium ($\chi'' \equiv 0$) and when it is not in equilibrium ($\chi' \equiv 0$).

**Magnetic system in equilibrium – low frequency regime**

The two Lagrange equations, having chosen as generalized variables the physical phase lag and the induced magnetization, are:

$$\frac{\partial U}{\partial (\varphi_f - \varphi_c)} + \frac{\partial U}{\partial (\omega_f - \omega_c)} = 0; \quad \frac{\partial U}{\partial \varphi_i} + \frac{\partial F_R}{\partial \omega_i} = 0$$  \hspace{1cm} (A.6)

Evaluating (A.6) we obtain the following set of coupled equations:

$$\frac{d\varphi_c}{dt} = \frac{(\chi')^2 \omega_0 H^2}{168 \eta_{\text{eff}}} \sin[2(\varphi_f - \varphi_c)] + \frac{m_{\mu_0 \omega_0 H}}{14 \pi \eta_{\text{eff}} R^3} \sin(\omega_f t - \varphi_c + \theta) + \frac{2 \mu_0 H^2}{21 \eta_{\text{eff}}} \chi''$$  \hspace{1cm} (A.7)

$$\sin(\varphi_f - \varphi_i) + \frac{1}{6} \frac{(\chi')^3}{(\chi')^2 + (\chi'')^2} \sin[2(\varphi_f - \varphi_c)]$$  \hspace{1cm} (A.8)

In this frequency regime the complex part of the susceptibility is negligible, thus $\chi'' \equiv 0$, and (A.7) and (A.8) simplify (3.10) and (3.11).
Magnetic system out of equilibrium – high frequency regime

The magnetization lags behind the field when the relaxation time of (part of) the grains is comparable with the period of the external field. The fast dynamics implies that there are losses due to remagnetization of the magnetic material. The infinitesimal power $dP$ that is absorbed can be derived from Maxwell's equations:\(^\text{(30)}\):

$$dP = \vec{H} \cdot \frac{\partial \vec{B}}{\partial t} \, dtdV \quad (A.9)$$

Since $\vec{B} = \mu_0(\vec{H} + \vec{M})$, we can identify two terms: one relative to the power absorbed by the magnetic material and one relative to the work necessary to sustain the field. We will neglect the second term since it is taken into account by the current in the coils. Regarding the absorption term we have to consider that the material opposes to being magnetized, which can be described by a demagnetization factor $\alpha$. The magnetic induction inside the material equals $\vec{B} = \mu_0(\vec{H}_{\text{ext}} + (1 - \alpha)\vec{M})$. For a prolate ellipsoid (which, as a first approximation, is descriptive of a two-particle cluster) the magnetic work is a function of the external fields\(^\text{(29)}\) and we need to solve the following integral in order to estimate the power absorbed in the material:

$$P_a = \mu_0 \int_V \int_0^M \vec{H} \cdot d\vec{M} = \int_V \int_0^{T_f} \vec{H} \cdot \frac{\partial \vec{M}}{\partial t} \, dt \quad (A.10)$$

A magnetic field $\vec{H}$, rotating in the $xy$ plane, can be written in complex notation:

$$\vec{H} = \text{Re} \left\{ H \left( \begin{array}{c} 1 \\ 0 \end{array} \right) e^{i\omega t} \right\} \quad (A.11)$$

and the magnetization is then $\vec{M} = (\chi' - \chi'')\vec{H}$. The time differential of the magnetization is:

$$d\vec{M} = \chi \frac{\partial \vec{H}}{\partial t} \, dt = \text{Re} \left\{ i\omega_1 H \chi' \left( \begin{array}{c} 1 \\ 0 \end{array} \right) e^{i\omega t} + \omega_1 H \chi'' \left( \begin{array}{c} 1 \\ 0 \end{array} \right) e^{i\omega t} \right\} \quad (A.12)$$

Evaluating (A.12) the in-phase component vanishes and only the high frequency term described by the complex susceptibility is left. The power absorbed in the material becomes:
\[ P_a = \mu_0 V \chi'' \omega_t H^2 \]  \hspace{1cm} (A.13)

which, for the particles used in these experiments, is of the order of $10^{-14}$ W. The frequencies at which the absorbed power becomes relevant are of the order of $100 \text{ KHz} - 1 \text{ MHz}$, but on the other hand the cluster is rotating only at a few Hz. The power spent to overcome the viscous drag and rotate the cluster is proportional to the Rayleigh dissipation function: \[ P_r = 28\pi \eta_{eff} R^3 \omega_c^2, \]
which implies that \[ P_{diss} = P_a - P_r. \] For typical values of \( \omega_f = 100 \text{ KHz}, \eta_{eff} = 10^{-3} Pa \cdot s, \omega_c = 1 Hz, R = 0.5 \mu m, \mu_0 H = 10^{-3} \text{ mT} \) and \( \chi'' = 0.5 \), the ratio between the power used to rotate the cluster and the power dissipated in heating is of the order of $10^{-5}$. This dissipation affects implicitly the equation of motion of the cluster, since the power available to rotate and overcome the viscous drag equals the difference between the absorbed and dissipated power.

The two-particle cluster is able to spend only a small percentage of the absorbed power to rotate and overcome the viscous drag. The power that is actually used is given by (A.4), and (A.7) is still representative of the motion of the cluster at high frequency. We consider an infinitesimal interval of time \( dt \) large enough to have an infinitesimal change in the angle of the cluster \( d\varphi_c \). During \( dt \) the field and the induced magnetization are able to perform several rotations and only the average effect of the rotation is experienced by the cluster (and the permanent moment). This is the reason why dipole–dipole interaction and the coupling with the permanent moment vanish at high frequency and (A.7) simplifies to:

\[ \frac{d\varphi_c}{dt} = \frac{2\mu_0 H^2}{21\eta_{eff}} \chi'' \]  \hspace{1cm} (A.14)

As previously noted (A.8) shouldn't be valid anymore, since it was derived imposing equilibrium. If we indeed consider that \( \chi' = 0 \), (A.8) becomes \( \sin(\varphi_i - \varphi_f) \). If (A.8) would hold even in the high frequency regime, it would imply that the induced moment and the field are parallel, resulting in zero torque acting on the cluster. At high frequencies the magnetic system is indeed in an out-of-equilibrium situation and the constraint (A.8) is not valid.
References

21. Out-of-plane rotations were rarely observed in our experiments. It was observed on some two-particle clusters in the condition of low fields with frequencies close to the critical frequency. We attribute such behavior to the presence of a significant off-axis permanent moment in the two-particle cluster.
Frequency-selective rotation of nanoparticle clusters for rapid and sensitive detection of biomolecules

Abstract. We describe an opto-magnetic bionanotechnology for rapid and sensitive solution-based affinity assays. Clusters made from bio-active magnetic nanoparticles undergo rotational motion in the volume of a fluid under frequency-controlled magnetic actuation. The clusters show a time-dependent scattering cross-section to an incoming light beam. We demonstrate that the temporal behavior of the scattered light intensity relates to the number, the magnetic properties and the size distribution of the clusters, independently revealing the average value and variation in the magnetic properties of the nanoparticles as well as the concentration of clusters. The method is applied to detect a model protein (biotinylated BSA) in fluid by inter-nanoparticle binding. In a total assay time of less than 3 minutes, we demonstrate a limit of detection lower than 400 fM in buffer and 5 pM in human plasma.

This chapter is based on Ranzoni et al., Nano Letters, 2011, 11 (5), 2017-2022.
4.1 – Introduction

The need for pervasive healthcare drives the field of in vitro diagnostics towards point-of-care solutions to detect biomolecules\textsuperscript{1-5}. The technological requirements are very demanding, since point-of-care testing needs to be as reliable and quantitative as testing in a centralized laboratory and at the same time a faster response is required (in the order of a few minutes) in a cost-effective, portable and integrated detection platform that operates on a small sample volume\textsuperscript{6}.

Affinity assays make use of biological molecules to capture specific target molecules from a sample and allow a determination of their concentration. Affinity capture is very effectively achieved by dispersing nano- or micro-particles coated with capture molecules into a sample volume\textsuperscript{7-9}. Rapid and efficient capture results from the high surface-to-volume ratio of the particles and the high effective concentration of capture molecules in the fluid volume. Ideally, the volume-based capture process is directly followed by a volume-based detection of the captured target molecules. However, it is very challenging to conceive a solution-based detection principle that is very sensitive and specific, and that does not intrinsically suffer from large background signals from the volume that is probed.

Since a few years superparamagnetic particles are being investigated for a novel generation of all-volume-based assays. In the volume of the fluid, magnetic particles capture target molecules and the formation of target-induced clusters of particles is detected. Baudry et al.\textsuperscript{10} have demonstrated that a magnetically-induced arrangement of nanoparticles in chains results in a very rapid formation of target-induced nanoparticle clusters, which they quantified by a decrease of the optical transmittance of the sample. Other groups measured cluster formation by the reduction of thermal nanoparticle diffusion\textsuperscript{11}, by monitoring the growth of long chain lengths\textsuperscript{12}, or by a relative change of the transverse relaxation time ($T_2$) in nuclear magnetic resonance\textsuperscript{13}. All these reported physical measurement principles have in common that they are not intrinsically selective to two-particle clusters with respect to single particles. Single particles already generate a large baseline signal and changes with respect to the baseline quantify the presence of two-particle clusters. The fact that a small change of signal is measured on a large background limits the ability to resolve few particle clusters in the midst of a high number of single nanoparticles, and thereby also limits the sensitivity and rapidity of the assays.
4.2 – Opto-magnetic cluster immunoassay

Here, we demonstrate a novel technique to selectively actuate, characterize and detect clusters of magnetic nanoparticles within an all-volume-based biological assay. The experimental arrangement is sketched in Figure 4.1. A laser beam collimated along the $\zeta$-axis illuminates a glass cuvette. Four electromagnets induce a rotating magnetic field inside the cuvette, which causes the magnetic clusters to rotate in the $xz$-plane. A photodetector collects light that is scattered along an angle of approximately 30 degrees from the $\zeta$-axis. Figure 4.1b describes the different phases of the assay. A short incubation, allowing efficient capture of the target proteins, is followed by the application of a magnetic field to induce chain formation. In the chains the nanoparticles interact and rapidly form inter-nanoparticle bonds via the captured target molecules. Thereafter the field is removed to allow the chains to disassemble. Finally, a rotating magnetic field is applied that selectively actuates the clusters for detection.

The sensitive and selective detection of two-particle clusters embedded in an ensemble of single nanoparticles is based on two distinguishing features, namely magnetic anisotropy and optical anisotropy. The magnetic shape anisotropy of a two-particle cluster enables frequency-controlled rotation\textsuperscript{14}, while the optical anisotropy of a cluster generates a modulation of optically scattered light. Single particles contribute negligibly to the optical modulation because they lack the characteristic magnetic and optical anisotropies of the two-particle clusters. Figure 4.1c shows the measured optical scattering of clusters in a field of $\mu_0H = 3.5 \text{ mT}$ rotating at a frequency $\omega_f/2\pi = 1 \text{ Hz}$. We observe that the signal period equals half the period of the applied field. This is a direct consequence of the equivalence of individual particles and the resulting point symmetry of a two-particle cluster. The data show that scattering is highest when the clusters are aligned perpendicular to the optical beam, i.e. when they expose their largest geometrical cross-section toward the incoming light beam. The orientations of lowest signal are close to an orientation along the optical beam. Figure 4.1d shows the calculated geometrical cross-sectional area as a function of $\phi_c$, the angle of the cluster axis to the $\zeta$-axis, for a cluster that consists of two nanoparticles with radius $a$. The geometrical cross-sectional area reproduces the half-period characteristic and has the same phase as the optical scattering signal, but the shapes of the curves are quite distinct. For example, the measured scattering curve shows interesting subtle features when
Fig. 4.1) The opto-magnetic system and nanoparticle assay. Panel a sketches the optomagnetic platform used in our experiments. A collimated laser beam is focused at the center of four electromagnets where a glass cuvette is placed. The light scattered at an angle of approximately 30 degrees with respect to the incoming laser beam is focused onto a photodetector. Figure 4.1b shows the three phases of the biological assay. First, biologically-activated nanoparticles are incubated with the target proteins. Thereafter a rotating magnetic field is applied to drive the formation of nanoparticle chains, which enables effective inter-nanoparticle binding. Finally, the magnetic field is removed to allow unbound nanoparticles to redisperse, and the optical scattering is detected under frequency-selective magnetic actuation. Figure 4.1c shows the typical optical scattering signal measured from two-particle clusters in a magnetic field rotating at 1 Hz. Figure 4.1d shows the calculated geometrical cross-section of a two-particle cluster during the rotation.
the clusters are nearly aligned along the optical beam ($\varphi \sim n\pi$). We attribute such features to the angle-dependent nature of the differential scattering cross section $\sigma_c(\vartheta, \varphi)$ of the clusters\textsuperscript{15, 16}, a topic that we will address in further research.

### 4.3 Results and Discussion

In order to calibrate the opto-magnetic detection system, we performed experiments for different solution concentrations (see Figure 4.2a). A stock solution was diluted to a particle concentration of 2 mg/ml and sonicated, leading to a solution with many single nanoparticles and a low number of two-particle clusters. The composition of the calibration sample was quantified by optical microscopy, showing a 1:20 ratio of two-particle clusters to single nanoparticles. Clusters of larger size were not observed. The recorded curves of optical signal as a function of time were analyzed by an FFT algorithm (Fast Fourier Transform) with an integration time of 3 seconds. The FFT spectrum (see inset) shows only even harmonics, as expected from the point symmetry of the clusters. The peak at $2f$ dominates the spectrum. The magnitude of the $2f$ peak shows a linear dependence on the particle concentration, with a dynamic range of about two decades. From the slope of the curve, the known concentration of two-particle clusters in the solution, and the optical probing volume in our system (about 1 nL), we deduce a value of $0.27 \, \text{V}/\sqrt{\text{Hz}}$ for the optical signal per two-particle cluster in our setup.

Our system allows a detailed characterization of the magnetic properties of the clusters. In a recent paper\textsuperscript{14} we have developed the equation of motion for a single two-particle actuator in a rotating magnetic field. In the low-frequency regime, the clusters rotate synchronously with the applied field. At the critical frequency, the phase difference between the applied field and the magnetic moment is maximum, so the applied torque and rotational frequency are also maximized. Beyond the critical frequency, the rotation shows a wiggling behavior in which forward and backward motions alternatingly appear. The backward rotations reduce the net forward angular velocity, an effect that becomes stronger for increasing frequency of the external field. When magnetic shape anisotropy dominantly generates the magnetic torque, the equation describing the motion of a two-particle cluster in a uniform magnetic field $\vec{H}$ rotating in the $xz$ plane at frequency $\omega_r$, is given by:
Fig. 4.2) Optical scattering signal as a function of particle concentration and magnetic field properties. The ratio of two-particle clusters to single particles is about 1:20. The linear behavior in panel a shows that the signal is proportional to the number of clusters present in the sample and allows us to estimate the signal per two-particle cluster. The inset shows the Fourier transform of the signal measured at a particle concentration of 250 μg/ml and a field frequency of 5 Hz. Panel b shows the frequency dependent response of particles with a diameter of 500 nm for several values of the strength of the magnetic field. The crossing point of the linear fits at low and intermediate frequencies gives the value of the critical frequency (the lines are shown for the measurement at 7.5 mT). The inset shows the value of the critical frequency as a function of the magnetic field strength; the quadratic fit demonstrates that the dipole-dipole interaction is the main source of the magnetic torque. Panel c shows the same data as in Figure 4.3b, but now plotted as function of the dimensionless Mason number. The low frequency (LF), intermediate frequency (IF) and high frequency (HF) zones are indicated. To fit the data, we numerically solved equation (1) for an ensemble of 100 clusters with a normal distribution of volume susceptibility. The mean value of the distribution was chosen to equal the average value obtained by the measurements of critical frequency shown in the inset of Figure 4.2b; the standard deviation was varied to best fit the experimental data by minimizing the mean square error. The data of the 500 nm diameter Masterbeads give a volume susceptibility of 2.4±0.8. The data of the 300 nm diameter Bioadembeads (see inset) give a volume susceptibility of 2.0±0.9.

\[ \frac{d\varphi_c}{dt} = \omega_{\text{crit}} \sin(2(\varphi_i - \varphi_c)), \quad \text{with} \quad \sin(\varphi_i - \omega_f t) + \frac{\chi}{16} \sin(2(\varphi_i - \varphi_c)) = 0 \quad (4.1) \]

where \( \omega_{\text{crit}} = \frac{\mu_0 \chi^2 H^2}{168 \eta} \) represents the value of the critical frequency, \( \varphi_i \) is the angle between the direction of the induced magnetic moment and the \( \zeta \)-axis, \( \varphi_c \) is the angle between the axis of cylindrical symmetry of the cluster and the \( \zeta \)-axis, \( \mu_0 \) is the magnetic permeability of vacuum, \( \chi \) is the dimensionless volume susceptibility of the magnetic nanoparticle material, and \( \eta \) is the
viscosity of the fluid medium. The equations are derived by balancing the magnetic and viscous torques. The equations are independent of the size of the nanoparticles because the magnetic and viscous torques both scale with the volume of the particles; this means that our actuation method is in principle applicable to a wide range of particle sizes.

Figure 4.2b shows the frequency-dependence of rotation of the clusters for different magnitudes of the applied magnetic field, measured on a mixture of two-particle clusters and single particles. In the low-frequency regime, the signal is independent of frequency since the clusters rotate synchronously with the applied field. At intermediate frequencies a gradual decrease of signal is observed. We attribute the signal decrease to a progressive diminishment of the number of two-particle clusters that is able to rotate synchronously with the magnetic field. A spread in size and magnetic content in the nanoparticles results in a distribution of critical frequencies; the clusters with the lowest volume susceptibility are the first to deviate from the synchronous rotation and at higher frequencies more and more clusters enter the regime of wiggling rotation. In the wiggling regime, the amplitude of the 2f modulation decreases and FFT signals appear at lower frequencies. We determined the critical frequency from the point where the intermediate frequency curve extrapolates to unity, as indicated in Figure 4.2b. The inset shows the measured critical frequency as a function of the applied field; the observed quadratic dependence proves that the magnetic shape anisotropy of the clusters is at the origin of the rotation.

The data can also be expressed as a function of a dimensionless parameter, the Mason number, which represents the ratio between viscous and magnetic torque:\(^{(14)}\)

\[
Mn = \frac{16\eta\omega}{\mu_0\chi^2H^2} \quad (4.2)
\]

At the critical frequency (see equation 4.1) the Mason number equals unity. In Figure 4.2c the data for nanoparticles with a diameter of 300 nm and 500 nm are plotted as a function of the Mason number. The measurement points collapse into a single curve that is specific for the type of particle. We have modeled the curves by summing responses for an assumed normal distribution of susceptibility values. For the 500 nm particles a good curve fit is found with \(\chi = 2.4\pm0.8\), in agreement with the value of 2.65 found by Vibrating Sample Magnetometry (VSM). For the 300 nm particles, the curve fit yields
Fig. 4.3) Dose-response curves for assays in buffer (panel a) and in plasma (panel b). For every measurement point a frequency scan was performed as in Figure 4.2b, measured for a field magnitude of 3.5 mT. The signal corresponds to the low-frequency plateau value (1 to 5 Hz) of the 2f signal of the FFT spectrum. The dashed lines are guides to the eye. In panel a, the final nanoparticle concentration was 85 μg/ml. The signal level at low concentrations corresponds to approximately 20 two-particle clusters in the optically probed volume. The dashed lines show two slopes which reflect the cluster size distribution, as is further detailed in figure 4.4. In panel b, the final particle concentration was 55 μg/ml; the signal at low concentrations corresponds to the presence of roughly 50 two-particle clusters in the probing volume. We attribute the higher blank values in plasma compared to buffer to the presence of interfering agents in the complex matrix. The grey lines in both panels represent the value of $S_b + 3\sigma_b$ for the blank measurements.

\[ \chi = 2.0 \pm 0.9 \], which compares well with the VSM value of 2.15 and with data from confined Brownian motion analysis\(^{17}\).

In the above experiments we have demonstrated that optical scattering is an accurate tool to characterize the rotational dynamics of an ensemble of two-particle clusters and that the amplitude of the 2f signal is an accurate measure for the amount of clusters in the sample. We proceed by investigating assays as in Figure 4.1b, using strepavidin-coated magnetic nanoparticles and biotinylated BSA (bBSA) as target molecule. A 6 μl sample of magnetic nanoparticles and bBSA is incubated for 10 seconds. The sample undergoes magnetic chaining for 2 minutes and is then diluted to tune the signal from the nanoparticles to the dynamic range of the photodetector and to avoid potential cluster growth during the subsequent detection phase. Detection is performed under frequency-selective magnetic actuation. Further details are given in the Material and Methods section.

Figure 4.3 shows dose-response curves for assays in buffer and in plasma. The opto-magnetic signal clearly increases as a function of the target concentration. Interestingly, the dose-response curve in buffer shows two distinct slopes, sketched with dotted lines in the figure. We attribute the change of slope to a transition in the size distribution of the clusters. The size
Fig. 4.4) Frequency response for three concentrations of bBSA in buffer (0.63 pM in panel a, 3.15 pM in panel b, and 250 pM in panel c). The measurements were performed in a field of 3.5 mT with an averaging time of 3 seconds. The critical frequency shifts to lower values for increasing bBSA concentrations due to the presence of clusters of increasing size. The signal at low frequencies increases with the concentration of antigen because of the larger size and number of clusters. The dotted lines are obtained by fitting the experimental points and are used to estimate the critical frequencies. The data at a concentration of 3.15 pM show the co-presence of clusters made of two and three nanoparticles, respectively characterized by a critical frequency of approximately $\omega_{\text{crit}}/2\pi = 7$ Hz and $\omega_{\text{crit}}/2\pi = 16$ Hz. At the latter critical frequency, the slope of the frequency dependent signal doubles, which indicates the co-presence of larger (triplets) clusters in the sample.

During incubation, the nanoparticle concentration is approximately 10 pM. So at target concentrations below 2 pM only two-particle clusters are statistically likely to form. When the number of bBSA molecules increases and becomes comparable to the number of nanoparticles, the probability increases that clusters consist of more than two nanoparticles. To further investigate the concentration dependence, we measured frequency response curves for three concentrations of bBSA, as shown in Figure 4.4. The critical frequency is derived from the crossing between the fits at low and intermediate frequencies. The critical frequency is about 13 Hz for a target concentration of 0.6 pM, reduces to 7 Hz for 3.1 pM, and becomes 4.8 Hz for 250 pM. In fact, the curve at 3.1 pM shows two critical transitions, with
increasing slope steepness. We attribute the two slopes at 3.1 pM to the contemporary presence of comparable quantities of two-particle and three-particle clusters. The dependence of the critical frequency on the number of particles in a cluster has been theoretically investigated\textsuperscript{18} and it is in agreement with our results. The low-frequency concentration-dependent signals lead to a dose-response curve as in Figure 4.3a. The detection limit, defined as the level where the signal equals \( S_b + 3\sigma_b \), with \( S_b \) is the average of the blank signal and \( \sigma_b \), the standard deviation of the blank signal, is found to be below 400 fM. The detection limit is determined by non-specific binding processes of the nanoparticles. The signal saturates at a concentration of about 100 pM, caused by the limited number of nanoparticles available for cluster formation. Analytical assays are particularly challenging in complex biological matrices such as blood plasma, due to the large quantities of potentially interfering molecules\textsuperscript{19}. Figure 4.3b shows a dose-response curve measured in human plasma. The optical signal increases with the concentration of antigens and reaches saturation at a value of approximately 100 pM. A transition of slope as is observed in buffer is not seen in plasma. The reason is that the blank levels are higher in plasma, due to the presence of interfering agents that generate non-specific binding between nanoparticles. The blank level has variations of about 13\%, which gives a value close to 5 pM as the limit of detection. To our knowledge, this is the first literature report of a dose-response curve with low-picomolar protein detection in undiluted plasma, for an all-volume-based cluster assay using magnetic nanoparticles.

4.4 – Conclusions

In this chapter, we have demonstrated an optomagnetic technology that is suited to characterize nanoparticle properties and to sensitively and rapidly detect biological molecules in a very small sample volume. A measurement of the frequency response reveals the magnetic properties, concentration and size distribution of clusters in the sample. Frequency-selective cluster rotation provides a baseline-free detection technique for solution-based biological assays in buffer and in human plasma. We have demonstrated a limit of detection of 400 fM in buffer and 5 pM in plasma, in a total assay time of less than 3 minutes. The platform integrates two bio-orthogonal principles, namely magnetic actuation and optical detection, allowing for independent tuning of the experimental parameters. In the optical
domain, the technology holds promise for further studies of the angle-dependent scattering properties of magnetic nanoparticles, with high accuracy due to magnetic control of orientation and the possibility to average signals over several revolutions. In the field of colloid interactions, the technology will enable studies of the kinetics of colloidal interactions for a wide variety of systems, for example nanoparticles with different surface chemistries and in different matrices. Furthermore, the volume-invariance of the rotation dynamics (see Eq. 1) allows an extrapolation of the method toward smaller sizes of nanoparticles. The use of smaller particles will allow the characterization of novel nanoparticle materials, studies of their aggregation properties, as well as studies of all-volume-based biological assays with high nanoparticle concentrations and high assay kinetics. In summary, we have presented an innovative and versatile opto-magnetic bionanotechnology that paves the way for a wide range of nanotechnological and biochemical studies.

4.5 – Materials and Methods

Figure 4.1 sketches the experimental setup. A collimated laser beam is focused with a low numerical aperture lens (NA=0.025) into the center of a glass cuvette of square cross section. The low numerical aperture lens guarantees a depth of focus of 1 mm. The depth of focus is comparable to the optical path inside the cuvette (1 mm). The beam waist is calculated to be approximately 32 μm in diameter. Consequently the optically probed volume is approximately 1 nl. Nanoparticles of 300 nm (Streptavidin coated Bio-AdemBeads, AdemTech) were measured with a blue laser (405 nm, Nichia NDV4212T, operating at 120 mW). Nanoparticles of 500nm (Streptavidin coated Masterbeads, AdemTech) were measured with a red laser (658 nm, Sanyo DL-6147-240, operating at 40 mW).

The focus of the laser beam and the glass cuvette are placed in the center of a quadrupole electromagnet, which generates a rotating magnetic field in a vertical plane. The electromagnets have been calibrated with a Hall probe and generate a maximum field of 70 mT. A measurement of the frequency response of the magnets shows that the self-inductance of the coils becomes important only at frequencies above several hundreds of Hz. We measured the scattered light at an angle of roughly 30 degrees from the main optical axis, since it was found that this configuration maximizes the intensity. The detection path consists of a lens focusing the scattered light onto a
photodetector (New Focus, model 2031, gain 2.10\(^6\)). Voltage signals measured by the photodetector are sampled at 1 kHz during 3 s and stored in a file using digital data acquisition (National Instrument NI-DAQ 6259). The data are processed by an FFT algorithm in MATLAB to compute the signal amplitudes. The FWHM value of the 2f peaks is about 50 mHz.

The optical response of the system was investigated with a calibration sample. Nanoparticles from the stock solution were diluted to a concentration of 0.1 mg/ml in PBS buffer (10mM, pH 7.4) containing 5% w/v BSA (both purchased from Sigma-Aldrich). The sample was sonicated for 3 s with a sonic needle, operating at 40 KHz and 50 W. The solution viscosity, measured with a MCR300 rheometer Antoon Paar Physica, is 2.32±0.09 Pa.s. The samples have been examined under a microscope and the ratio between the number of two-particle clusters and the number of single particles was determined to be approximately 5%; no larger clusters could be identified in significant proportion (less than 0.1% of the total population).

When performing an assay, the nanoparticle stock solution is diluted to 2 mg/ml in buffer and the solution is exposed for 3 s to ultrasound at 40 kHz and 50 W to minimize the number of clustered nanoparticles in the initial sample. A 3 μl volume of streptavidin-coated nanoparticles is added to 3 μl of biotinylated BSA (bBSA, Sigma Aldrich, cod. A8549), for end-concentrations between 60 fM and 10 nM. Nanoparticles and bBSA are incubated for 10 s. Thereafter, during the magnetic chaining phase, the sample is exposed to a 5.3 mT field rotating at 1 Hz for 2 minutes. Prior to the detection step, the solution is diluted with de-ionized water to 85 μg/ml, because that gives a blank value approximately ten times larger than the instrumentation noise. The optical response to a frequency sweep is measured and each experimental point is the result of a 3 s averaging time with a field strength of 3.5 mT. The samples have been probed with frequencies between 1 Hz and 25 Hz. For experiments in human plasma, the nanoparticles in the 2 mg/ml solution are attracted to the bottom of a vial with a permanent magnet, the supernatant is removed and replaced by an equal volume of spiked human plasma. Plasma is taken from a pure human heparin plasma pool from 20 healthy donors (purchased from Innovative). All samples were prepared by spiking whole plasma with 30 μM bBSA in PBS buffer, and by subsequent dilutions in whole plasma to arrive at the required target concentrations for the dose-response curve. Consequently, the amount of PBS buffer in the final samples is negligible. The actuation protocols for chaining and detection are the same as
for the assay in buffer. Prior to detection, the plasma sample is diluted to a final nanoparticle concentration of 55 μg/ml, because that gives a blank value approximately ten times larger than the instrumentation noise. All points in the dose-response curves were measured in triplicate.
References


Single-step magnetic nanoparticle assay for biomarker detection with high speed and sensitivity directly in blood plasma

A long standing challenge in bioanalytical science is to detect low biomarker concentrations directly in complex biological matrices, without any separation or fluid manipulation steps. In this paper we demonstrate a homogeneous one-step immunoassay technology in blood plasma based on antibody-coated magnetic nanoparticles. Pulsed magnetic fields are used to trigger and detect biomarker-induced binding between nanoparticles. We demonstrate dose-response curves for Prostate Specific Antigen (PSA) measured directly in undiluted human blood plasma with a detection limit of 400-500 femtomol/L, in a total assay time of 14 minutes and an optically probed volume of 1 nL. We explain the dose-response curves with a model based on discrete binding of biomarker molecules onto the nanoparticles, which allows us to independently and quantitatively extract the reaction parameters for the binding of biomarker molecules onto the nanoparticles and for the biomarker-induced binding between nanoparticles. The demonstrated analytical performance and quantitative understanding of our versatile bionanotechnology renders it of interest for a wide range of applications in quantitative biology and medical diagnostics.

This chapter is based on Ranzoni et al., submitted.
5.1 – Introduction

Assay technologies capable of detecting minute concentrations of biomolecules are a fundamental driver for discoveries in biological research and applications in medical diagnostics. A common strategy to achieving a high detection sensitivity is by passing a biological sample through a series of process steps, e.g. sample dilution, affinity capture, washing, labeling, and chemical or biochemical amplifications. Disadvantages of a multi-step strategy are that every step increments time, requires reagents, involves fluid manipulation and introduces variability. As a result, multi-step assays generally take hours and the deployment into lab-on-a-chip devices is very complicated.

An opposite approach is to conceive an assay technology that is based on only a single biofunctional probing reagent. The probing reagent is spiked into the sample volume and biomarker detection follows by purely physical means, without any further additions or separations of chemical or biochemical materials. Nanotechnological principles that may enable such a homogeneous one-step assay are for example biomarker-induced conformational changes in molecular probe molecules, biomarker-induced binding between molecules or between nanoparticles, or conformational changes combined with inter-particle binding. Conceptually a homogeneous one-step assay is ideal for rapid measurements in small sample volumes, however, it is very difficult to conceive an underlying assay principle that can give a high sensitivity in complex biological matrices such as undiluted blood plasma. In fact high-sensitivity biosensing is much more complex than the proverbial finding of a needle in the hay stack, particularly for samples such as blood plasma with its very high levels of endogenous proteins.

In this paper we describe a homogeneous one-step assay technology based on pulsed magnetic nanoparticles. We demonstrate that the assay can be operated in undiluted human plasma with high sensitivity, and that the underlying bionanotechnological processes can be quantitatively analyzed based on discrete molecular and nanoparticle binding events. The assay starts by the spiking of a nanoparticle probing reagent into the sample. The nanoparticles are decorated with two types of antibodies, targeting different epitopes on the biomarker protein. After the spiking the assay proceeds in three phases as sketched in Fig. 1a. In the first phase the biomarker molecules are captured by the antibody-coated magnetic nanoparticles in diffusive random motion, in absence of a magnetic field. Thereafter a pulsating magnetic
Fig. 5.1) Concept of the assay technology based on pulsed magnetic nanoparticles. Panel (a) sketches the three phases in the assay (incubation, pulsation and detection) and the corresponding magnetic field. During pulsation, periods of nanoparticle concentration ($t_{\text{conc}}$) and nanoparticle diffusion ($t_{\text{diff}}$) are alternated. During detection, the clusters are magnetically rotated and thereby generate a modulation of optical scattering. The result is a curve of optical scattering signal as a function of frequency (as sketched in the inset); the plateau reveals the number of clusters in solution while the critical frequency reveals the nature of the clusters and the viscosity of the sample \textsuperscript{19}. Panel (b) shows a scheme of the modular formation of the molecular architecture that we have engineered to suppress non-specific interactions in complex matrices. Antibodies with biotinylated PEG (polyethylene glycol) linkers are coupled to streptavidin-coated nanoparticles. Thereafter a second layer of linkers is added in order to surround the antibodies by a shell of linkers.
field alternatingly concentrates the particles in chains (during time \( t_{\text{conc}} \)) and lets the nanoparticles diffuse by Brownian motion (during \( t_{\text{diff}} \)). The diffusive motion randomizes the angular orientations of the nanoparticles and facilitates biomarker-induced inter-nanoparticle binding. The inter-nanoparticle binding gives clusters of nanoparticles, which are sensitively detected by optical scattering at applied magnetic rotation frequencies \(^{19, 20}\). Curves are measured of the optical scattering signal as a function of the field rotation frequency (see the inset of Fig. 1a) where the plateau signal reveals the number of clusters in solution and the value of the critical frequency \( \omega_{\text{crit}} \) reveals the size of the clusters, as we will detail later. In our experiments the three phases of the assay take a total time of 14 minutes, with 10 min for the capture phase, 2 min for pulsation and 2 min for detection.

### 5.2 – Pulsed magnetic actuation

We first investigate the binding dynamics of nanoparticles in a pulsating magnetic field. The assay is a sandwich immunoassay for Prostate Specific Antigen (PSA) and we use a standard bioconjugation method (EDC coupling chemistry) to couple antibodies to carboxyl groups on the nanoparticles; details are given in the Supporting Material. The time and field required for the arrangement of nanoparticles into chains depends on the particle susceptibility and the particle density \(^{21}\). In our experiments we use a nanoparticle concentration of 1 pM, which corresponds to an average inter-nanoparticle distance of 12 μm. The particles with a diameter of 500 nm concentrate into chains very rapidly \(^{22}\) and in a magnetic field of 50 mT a concentration time \( t_{\text{conc}} \) of 2 s is sufficient. The role of nanoparticle diffusion in the inter-nanoparticle binding process can be studied by varying the diffusion time \( t_{\text{diff}} \), as is presented in Fig. 2a. The figure shows the optical scattering signal due to nanoparticle binding as a function of the diffusion time for a total of 20 applied pulses. The data show a steep signal rise at short diffusion times and a slight decrease at larger values. We can understand the steep rise from the time that is required for the Brownian randomization of the nanoparticle orientations, which equals the time that is needed for translational diffusion by one particle diameter [55 ms for a 500 nm particle in water \(^{23}\)]. The rotational and translational randomization increases the biomarker-induced binding probability of the nanoparticles. The decrease of inter-nanoparticle binding at long diffusion pulse widths (\( t_{\text{diff}} > 4 \) s) can be understood from the outward
Fig. 5.2) Nanoparticle binding study as a function of magnetic pulse parameters. Panel (a) shows the optical signal as a function of $t_{\text{diff}}$, the time for diffusive Brownian motion. The pulse width for nanoparticle concentration is $t_{\text{conc}}=2$ s and the total number of pulses is 20. The main panel shows data for a biomarker concentration of 15 pM, the inset for 150 pM. Panel (b) shows the nanoparticle binding signal as a function of the pulsation time. The closed line is a Langmuir curve with an association time constant of 37 s. Panel (c) shows the critical frequency measured as a function of the biomarker concentration, for binding with pulsation ($t_{\text{diff}}=4$ s) and without pulsation ($t_{\text{diff}}=0$), in both cases for a field strength of 3.7 mT. The critical frequency is constant in the doublet regime (where $m=1$ and $m=2$) and drops as larger clusters appear (multiplet regime, $m>2$). The top x-axis indicates the cluster multiplicity number qualitatively observed by video microscopy. The inset shows a dose-response curve with pulsed nanoparticles, using a standard COOH surface chemistry. The horizontal line indicates the average value of the blank plus three times the standard deviation of the blank ($N=10$). The dose-response curve shows a detection limit of 5 pM in buffer.
diffusion of the nanoparticles which lowers the local nanoparticle concentration and thereby the encounter probability. We observe the same binding characteristics for different biomarker concentrations (see inset) which proves that the inter-nanoparticle binding dynamics are dominated by the properties of the nanoparticles and the applied field. Fig. 2b shows the nanoparticle binding as a function of the total pulsation time. The nanoparticles are concentrated into chains during the first few pulses and subsequently the binding signal rises and saturates. A Langmuir curve fit reveals a time constant of $\tau = 37\pm 10 \text{ s}$. The time constant is independent of the biomarker concentration (see inset) which demonstrates that we operate in a regime where the antibodies on the nanoparticles rather than the biomarker molecules are limiting the inter-nanoparticle binding reaction. From the time constant we can estimate an effective biomarker-induced inter-nanoparticle association rate of $k_{on} \approx 10^4 \text{ M}^{-1} \text{s}^{-1}$ (see the Supporting Material).

The inter-nanoparticle binding process generates an evolution of species in the fluid. The population starts with single nanoparticles. After biomarker capture and pulsation, two-particle clusters appear, denoted by the multiplicity number $m=2$. The formation of two-particle clusters goes at the cost of the number of single particles in solution. We speak of the doublet regime when the solution dominantly contains single particles and two-particle clusters ($m=1$ and $m=2$). We speak of the multiplet regime when clusters of higher multiplicity appear in solution ($m>2$). At any time, the total signal $S$ can be expressed as a summation over the species in solution: $S = \sum S_m N_m$, with $S_m$ the signal per cluster and $N_m$ the number of clusters with multiplicity $m$.

We can separate the doublet and multiplet regimes by recording the critical frequency of rotation. The critical rotation frequency $\omega_{\text{crit}}$ is the maximum applied rotation frequency that a cluster can synchronously follow (see the signal-versus frequency inset in Fig. 1a). The critical frequency depends on the cluster size; $\omega_{\text{crit}}$ is largest for $m=2$ and it decreases for $m>2$. Fig. 2c shows the critical frequency as a function of the biomarker concentration for an assay with and without pulsation. The development of the critical frequency is in agreement with observations of the cluster multiplicity by video microscopy (see top x-axis). For binding with pulsation, the transition from the doublet to the multiplet regime happens at a 2-3 times lower biomarker concentration than without pulsation, namely at a value of about 10 pM. The pulsation results in a dose-response curve with a detection limit of 5
pM in buffer (see inset). It appears that the system is in the multiplet regime over nearly the complete dose-response curve. So the standard bioconjugation method to couple antibodies to the nanoparticles generates a high level of non-specific binding and thereby masks the doublet regime in the dose-response data.

5.3 – Surface engineered molecular architecture

In order to be able to investigate samples with sub-picomolar biomarker concentrations in matrices of high biological complexity, we have developed a versatile molecular architecture as sketched in Fig. 1b. Antibodies with flexible biotinylated linkers and naked biotinylated linkers are attached to streptavidin-coated particles. The linkers shield the surface of the nanoparticles and enhance the colloidal stability. Thereafter another layer of linkers is added as an inert shell around the antibodies. We have studied linkers of different lengths and compared the modular architecture to the standard coupling of antibodies to carboxyl groups on the nanoparticles, showing a systematic increase of the hydrodynamic radius, a shift of the charge state, and a reduction of the non-specific binding (see Supporting Material). The surface architecture with linkers very effectively suppresses the formation of non-specific particle-particle bonds. The pulsed assay reveals a pronounced effect of the surface architecture on the biomarker-induced binding of nanoparticles, as is shown in Fig. 3. Panel (a) shows the critical frequency measured as a function of the biomarker concentration, in buffer and in untreated plasma. The surface architecture with linkers gives a doublet-to-multiplet transition at a concentration of about 3 pM in buffer, so at a three times lower concentration than with the standard carboxyl antibody coupling. We also see that a reliable low-concentration assay can now be performed in untreated plasma. The critical frequency in untreated plasma is about one third of the value in buffer because of the higher viscosity of plasma. Fig. 3b shows a dose-response curve recorded in buffer with the linker architecture on the nanoparticles. The non-specific inter-particle binding is very effectively suppressed and results in a limit of detection of 160 fM. The inset shows that the nanoparticles have a time constant for inter-nanoparticle binding of only 12.7±1.7 s, which is three times faster than with the standard carboxyl coupling of antibodies.

The shape of the dose-response reveals two segments, each characterized by signal growth followed by a plateau. Interestingly, the
concentration ranges of the two segments coincide with the ranges for the doublet and the multiplet regime (see Fig. 3a). We can understand the shape of the dose-response curve from a model for the nanoparticle binding processes in the fluid (see Supporting Material). The model is based on the fact the capturing of biomarkers by the nanoparticles is a discrete process governed by Poisson statistics. For low biomarker concentrations most nanoparticles do not carry a biomarker molecule, which gives a state with mainly single nanoparticles and only a few doublets. At high biomarker concentrations the binding probabilities increase, resulting in a state with many doublets and few single nanoparticles. For the doublet regime (with only $m=1$ and $m=2$) the total signal can be expressed as a function of the average number of biomarkers per nanoparticle $x$:

$$S(x) = \sum_{m=1}^{2} S^m_{cap} N^m_{cap} \approx S_2 \left[ N^m_{cap} + \left( N - 2N^m_{cap} \right) \frac{1-e^{-\xi x\Gamma}}{2-e^{-\xi x\Gamma}} \right]$$

where $S_2$ is the optical signal per doublet, $N^m_{cap}$ is the number of doublets not caused by biomarkers (non-specific doublet background), $N$ is the total number of nanoparticles, $\xi$ is the fraction of biomarkers on the nanoparticles that is sterically available to form an inter-nanoparticle bond ($0 \leq \xi \leq 1$), $\Gamma$ is the fraction of biomarkers that is captured from solution onto the nanoparticles (Langmuir kinetics, $0 \leq \Gamma \leq 1$), $C_b$ and $C_N$ are respectively the concentration of biomarkers and nanoparticles in the original solution, $t_{cap}$ is the duration of the biomarker capturing process, and $\tau_{cap}$ is the capture time constant. The steric availability factor $\xi$ takes account of the fact that captured biomarker molecules have a limited potential to generate an inter-nanoparticle bond, due to steric hindrance originating from antibody misorientation or surface roughness of the particles. Fig. 3b shows a fit of equation (1) to the data in the doublet regime. We have determined in an independent experiment that the captured fraction in buffer is about $\Gamma=0.95$. This allows us to deduce from the curve fit that the biomarker steric availability for the surface architecture with linkers is $\xi=0.43\pm0.12$, so about half of the captured biomarker molecules are sterically available to form an inter-nanoparticle bond. For the standard carboxyl coupling of antibodies, the steric availability is $\xi=0.26\pm0.07$ (see Supporting Material), which is nearly a factor two lower than for the surface
Fig. 5.3) Nanoparticle binding as a function of biomarker concentration, in buffer and in blood plasma. Nanoparticles are coated with the molecular surface architecture with linkers. The plasma samples including reagents and spiked PSA have a final plasma content of 95%. Panel (a) shows the critical frequency as a function of the PSA concentration for buffer and untreated blood plasma, measured with a field of 6 mT. Panel (b) shows the dose-response curve in buffer, where each point has been measured in triplicate. The doublet regime (up to 5 pM) has been fitted using equation (1); the curve fit in the multiplet regime is described in the Supporting Material. The inset shows the binding signal as a function of the pulsation time. The graphical sketch shows in green the effective areas for cluster growth, for single particles and for doublets. Panel (c) shows the dose-response curve in untreated blood plasma. The inset shows an overlay of the dose-response curves in buffer, precleared plasma and untreated plasma. The line is a curve fit according to the model description (see the Supporting Material). The horizontal line shows the level of the blank plus three times the standard deviation of the blank.

architecture with linkers. The transition from the doublet to the multiplet regime occurs at a biomarker concentration of about 5 pM. Interestingly the slopes of the doublet and the multiplet segments are the same, but the
A multiplet segment is shifted to higher biomarker concentrations, pointing to a reduced binding effectiveness of multiplets versus single particles. We attribute the shift to a reduced effective area for elongation of multiplets. In the doublet regime, the formation of clusters is driven by single particles, whose spherical shape implies that biomarker molecules over their complete surface area can induce inter-nanoparticle binding. In the multiplet regime, the process to grow doublets into longer multiplets is sterically hindered, because the doublets are magnetically oriented and only biomarkers captured at their extremities can generate further cluster elongation (see sketch in Fig. 3b). Consequently, the shape of a doublet causes a less effective biomarker-induced inter-nanoparticle binding process, which shifts the multiplet segment to higher concentrations in the dose-response curve. We can model the reduced binding effectiveness by an area ratio $\rho$, which is the ratio between the area of the two extremities and the total surface area of the cluster (see Supporting Material). From the fit we deduce an area ratio of $\rho=0.012\pm0.005$, which points to an effective binding cap with a height of $13\pm5$ nm. In Fig. 3c we show dose-response data for assays in blood plasma, namely for a mixed-gender plasma pool and for an untreated female plasma pool. The mixed-gender plasma pool was precleared by immuno-extraction of PSA originating from male plasma donors. Female plasma is by nature free of PSA, so PSA clearance was not required. The samples including reagents and spiked PSA have a final plasma content of 95%. The dose-response curve for the precleared plasma pool correlates perfectly with the dose-response curve in buffer across three decades of biomarker concentration (see inset). Measurements in untreated female plasma also correlate well but show a higher blank level due to non-specific binding. The limit of detection is 400-500 fM in both plasma types. A fit of the plasma dose-response data gives in plasma the same signal per doublet and the same effective binding cap as in buffer, but a slightly lower Langmuir factor ($\Gamma=0.72\pm0.25$) which we attribute to the higher viscosity of the matrix.

5.4 – Conclusions

We have described a novel homogenous one-step assay technology based on magnetic nanoparticles in a pulsating magnetic field. The bionanotechnology addresses the four scientific aspects that are essential for a homogeneous one-step assay: the capturing of low-concentration biomarker molecules is very efficient, the signal resulting from discrete biomarker binding
events is high, background signals from the probing volume are low, and non-
specific binding is small. The magnetic pulsation gives very effective
biomarker-induced inter-nanoparticle binding, and the detection by frequency-
selective rotation gives a high optical signal per inter-particle binding event
with an intrinsically low background level. We have demonstrated the ability to
record and model the population dynamics of the nanoparticle system, and the
ability to quantitatively extract the underlying parameters for capture of
biomarkers onto the nanoparticles and for biomarker-induced inter-
nanoparticle binding. The homogeneous one-step assay technology is highly
suited for rapid and high-sensitive protein analysis in complex biological
matrices. We have demonstrated a limit of detection of 400-500 fM for PSA
measured directly in human blood plasma, in a total assay time of 14 minutes
and an optical probing volume of only 1 nL. To our knowledge, the data
represent the first reported enablement of a homogeneous one-step
immunoassay in undiluted blood plasma, with sub-picomolar sensitivity in a
total assay time of minutes. The small probing volume, the flexible magneto-
temporal control of assay, the fact that separation and washing steps are not
necessary, and the quantitative understanding of the underlying assay processes
renders the technology suited for biological analysis and for integration into
lab-on-a-chip systems. Ultra small samples can be taken and analyzed for
biotechnological process monitoring 26 and libraries of biological materials can
be screened in lab-on-a-chip systems 27-29. The homogeneous one-step assay
technology is also suited for medical in-vitro diagnostics, particularly for rapid
point-of-need testing on small finger-prick sized samples 30-32. We envisage that
the novel bionanotechnology will lead to applications in quantitative biology
and medical diagnostics, thanks to the sensitivity, quantitative understanding,
rapidity, flexibility, and cost-effectiveness.

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5.5 – Supporting Material

Opto-magnetic platform. A collimated laser beam (658 nm wavelength, Sanyo DL-6147-240, operating at 15 mW) is focused with a low numerical aperture lens (NA=0.025) into the center of a glass cuvette of square inner cross section (1 mm$^2$). The low numerical aperture lens gives a depth of focus of 1 mm, equal to the optical path inside the cuvette. The beam waist diameter is calculated to be $w = 1.22\lambda/NA = 32$ µm. The optically probed fluid volume is approximately 1 nL. The focus of the laser beam and the center of the glass cuvette are placed in the middle of a quadrupole electromagnet, which generates a rotating magnetic field in a vertical plane [see A. Ranzoni et al., Nano Letters 11, p. 2017 (2011)]. The electromagnets have been calibrated with a Hall probe and generate a maximum field of 70 mT. The self-inductance of the coils becomes important only at frequencies above several hundreds of Hz. We measure the scattered light at an angle of about 30 degrees from the main optical axis, where the signal intensity is maximum. The scattered light is collected by a low NA lens onto a photodetector (New Focus, model 2031, gain 2.106). Voltage signals measured by the photodetector are sampled at 1 kHz during 3 s and stored in a file using digital data acquisition (National Instrument NI-DAQ 6259). The data are processed by an FFT algorithm in MATLAB to compute the signal amplitudes. The optical scattering signal from the clusters appears at twice the rotation frequency of the applied magnetic field, due to the point symmetry of the clusters. The FWHM value of the 2f peak is about 50 mHz. The precision of the 2f amplitude determination is better than 2%. Curves of the optical scattering signal as a function of frequency are determined between 1 and 25 Hz in a total scan time of 2 minutes. The signals are stable over time, indicating that no magnetically-induced clusters are generated during the frequency-selective detection phase and that only the chemically-formed clusters are measured.

Coating of COOH nanoparticles with α–PSA antibodies. Chemicals were purchased from Sigma Aldrich except if stated otherwise. EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, Thermo Scientific Pierce) is dissolved in de-ionized water at a final concentration of 40 mg/mL. 100 µL of COOH magnetic nanoparticles (AdemTech Masterbeads, 500 nm diameter) at 50 mg/mL are washed three times with 500 µL of MES buffer (50 mM MES buffer pH 6.2 + 0.001 [% v/v] Triton. The nanoparticles are suspended in 250
μL of MES buffer. The antibodies (α-PSA10 MAb and α-PSA66 MAb, purchased from Fujirebio) are added to the solution at a concentration of 20 μg per mg of nanoparticles and EDC at a final concentration of 2 mg/mL is added. The nanoparticles are incubated for 30 minutes with the antibodies at room temperature, mixed on a thermomixer at 600 rpm. The reaction is blocked by adding 2M Tris buffer pH 8.0 to a final concentration of 0.1 M. After 30 minutes the nanoparticles are magnetically washed twice and the supernatant is removed and replaced by 500 μL of 50 mM Tris buffer pH 8.0. As last step, the nanoparticles are suspended in 500 μL of storage buffer (AdemTech). The binding kinetics of PSA to the antibodies has been measured by surface plasmon resonance (BiaCore SPR instrument) and a value of \( k_{\text{on}} = 10^5 \text{M}^{-1}\text{s}^{-1} \) was determined. We can estimate an effective \( k_{\text{on}} \) for biomarker-induced inter-nanoparticle binding from the data in Fig. 2b. The data give a time constant of \( \tau = 37 \pm 10 \text{ s} \). From this time constant we can calculate an effective association rate \( k_{\text{on}} = 1/(\tau \cdot C) \), with \( C \) the effective concentration of antibodies during the pulsation phase. We have determined the number of active antibodies per nanoparticle by incubating known amounts of nanoparticles and PSA for 1 hour (long enough to reach equilibrium) and by measuring with UV absorption (Nanodrop) the amount of biomarker left in the supernatant. We found that each nanoparticle holds approximately 200 active antibodies. Thus the effective concentration of antibodies during the field-induced inter-nanoparticle binding process is approximately 200 per nanoparticle volume of about 500 nm cubed, so about 3 μM. This gives an estimate for the effective inter-nanoparticle association rate constant of \( k_{\text{on}} \approx 10^4 \text{M}^{-1}\text{s}^{-1} \).

**Coupling of PEG linkers to α-PSA antibodies.** Biotinylated PEG linkers modified with an NHS group (b-PEG-NHS, purchased from Creative PEG Works) were conjugated to the antibodies (α-PSA10 MAb and α-PSA66 MAb, purchased from Fujirebio). The concentration of antibodies was measured by UV absorbance (Thermo Scientific, Nanodrop). The b-PEG-NHS solution is prepared in PBS at 5 mM and is added to the antibodies in a 10 molar excess. The mixture is incubated for 1 hour at room temperature under gentle shaking. The biotinylated antibodies are purified through a PD10 column in 0.1% NaN₃ in PBS. Characterization of the antibodies demonstrates that 2 to 3 biotin groups are conjugated to each antibody.
**Linker multi-layer architecture on magnetic nanoparticles.** 10 mg/mL Streptavidin coated nanoparticles (500 nm Masterbeads, AdemTech, $6\times10^{10}$ particles per mL) are magnetically washed three times in PBS buffer and re-suspended in PBS by sonicating three times at 50 W for 3 s. 25 μg of biotinylated α-PSA antibodies per mg nanoparticles are added to the nanoparticles and incubated for 15 minutes under gentle shaking. Additional hydrolyzed b-NHS-PEG is added to the solution at a final concentration of 200 pmol per mg nanoparticles and incubated for 15 minutes under gentle shaking. An excess of streptavidin (2.2 nmol per mg nanoparticles) is added and incubated under gentle shaking for 10 minutes and then an excess of hydrolyzed b-NHS-PEG (90 nmol per mg nanoparticles) is added and incubated for 15 minutes under gentle shaking. The nanoparticles are magnetically washed and suspended in buffer (5% BSA in PBS with 1 mM biotin). The solution is sonicated 3 s at 50 W.

**Measurement of mean nanoparticle size and charge.** Measurements have been performed by dynamic light scattering (Malvern, Zetasizer). For measuring the hydrodynamic mean diameter, the nanoparticles have been diluted to 50 μg/mL in NaCl buffer and a sample volume of 1 mL was sonicated at 50 W for 3 s twice. The mean diameter progressively increases, from 506 nm (COOH) to 520 nm (Streptavidin) and to 550 nm (PEG coated nanoparticles). The same protocol has been followed for the zeta potential measurements, using 10 mM phosphate buffer pH 7.4. The measurements show a progressive reduction in the surface charge of the nanoparticles: carboxyl coated nanoparticles are most strongly charged ($Z=-23$ mV), then the particles with streptavidin ($Z=-19$ mV), and the particles with PEG linkers have the lowest charge ($Z=-14$ mV). In buffer, only the PEG coated nanoparticles showed no measurable non-specific inter-particle binding, which demonstrates that PEG linkers suppress non-specific interactions and increase the nanoparticle stability.

**Immunoassay samples.** We performed immunoassay experiments in three matrices, namely in buffer, in a mixed-gender plasma pool that was precleared of PSA, and in an untreated female plasma pool:
1) Experiments in buffer. PSA is spiked in buffer (5% BSA in PBS) from a PSA calibrator (60 μg/L) purchased from Fujirebio. In the immunoassay
experiments, 1.5 μL of α-PSA nanoparticles at 2 mg/mL (1:1 ratio of α-PSA10 MAb and α-PSA66 MAb nanoparticles) is added to 28.5 μL of buffered PSA.

2) Experiments in mixed-gender heparin plasma pool (purchased from Innovative). Mixed-gender plasma pools contain a background PSA level from male plasma donors. We cleared the plasma by incubating for 30 mins a volume of 0.5 mL of plasma with 5 mg nanoparticles coated with α-PSA antibodies and subsequently with 5 mg nanoparticles coated with Protein G to remove excess IgG. The nanoparticles are magnetically removed and the precleared plasma is spiked with PSA from the calibrator. The immunoassay capture starts by adding α-PSA nanoparticles to the sample, with 1.5 μL of nanoparticles at 2 mg/mL added to 28.5 μL of precleared plasma. The solution has a final plasma content of 95%.

3) Experiments in female heparin plasma pool (from SeraLab). Female plasma is by nature free of PSA, so PSA clearance is not required. The plasma is spiked with PSA from the calibrator and a detergent at 2% w/v in dry form. The immunoassay capture starts by adding α-PSA nanoparticles to the sample, with 1.5 μL of nanoparticles at 2 mg/mL added to 28.5 μL of plasma. The solution has a final plasma content of 95%.

Equations for cluster formation. We consider a system of N nanoparticles with concentration $C_N$ dispersed in a fluid with biomarker concentration $C_b$. The ratio $x = C_b/C_N$ represents the number of biomarkers divided by the number of nanoparticles in solution. The nanoparticle population is composed of two equal subpopulations (N/2) which bind to different epitopes on the biomarker molecules ($\varepsilon_1$ and $\varepsilon_2$). Therefore biomarker molecules can induce binding between nanoparticles of the two subpopulations by a molecular sandwich format. We assume that the two types of nanoparticles have equal association rates with the biomarker. The assay takes place in three phases: capture, pulsation and detection, as is indicated in Fig. 1. During the first phase the biomarkers are captured by the nanoparticles. In this section we want to develop an expression for the probability $P_1$ that a single nanoparticle has the potency to generate a biomarker-induced inter-nanoparticle bond, i.e. the chance that a nanoparticle has captured at least one biomarker molecule which is sterically accessible to generate inter-nanoparticle binding. The distribution of biomarkers over the nanoparticles is determined by Langmuir adsorption and Poisson statistics. Langmuir adsorption describes the fraction $\Gamma$ of
biomarker molecules that are captured onto the nanoparticles during the capture time $t_{\text{cap}}$:

$$
\Gamma = 1 - e^{-k_{\text{on}}C_{\text{Ab}}t_{\text{cap}}} = 1 - e^{-t_{\text{cap}}/\tau_{\text{on}}}
$$

(S.1)

with $k_{\text{on}}$ the association rate of biomarker molecules to antibodies, $C_{\text{Ab}}$ the volume concentration of antibodies, and $\tau_{\text{cap}} = 1/(k_{\text{on}}C_{\text{Ab}})$ the capture time constant. We can neglect biomarker dissociation ($k_{\text{off}}$) due to the short timescale of our immunoassays. The distribution of biomarker molecules $b$ ($b=0,1,2...$) over the nanoparticles is now given by Poisson statistics:

$$
p(b,\Gamma_{\infty}) = (\Gamma_{\infty})^b e^{-\Gamma_{\infty}} / b!
$$

(S.2)

and the probability that a nanoparticle has captured at least one biomarker molecule is given by:

$$
p(b \geq 1, \Gamma_{\infty}) = 1 - e^{-\Gamma_{\infty}}
$$

(S.3)

When biomarker molecules are captured onto the nanoparticles, not all molecules will have the potency to generate an inter-nanoparticle bond. For example, the surface of nanoparticles is generally rough on the molecular scale and a biomarker captured into a tight pocket may not be accessible for generating inter-nanoparticle binding. Also, a biomarker may be captured by an antibody which is misoriented and therefore not accessible for further binding. We define a steric availability factor $\xi$ ($0 \leq \xi \leq 1$) that describes the probability that a captured biomarker molecule is sterically able to generate inter-nanoparticle binding. Thus, the probability $P_i$ that a nanoparticle has binding potency is given by:

$$
P_i(\infty) = p(b \geq 1, \xi \Gamma_{\infty}) = 1 - e^{-\xi\Gamma_{\infty}}
$$

(S.4)

After the capture phase, the population of $N$ nanoparticles consists of four subpopulations: a nanoparticle targets either $\varepsilon_1$ or $\varepsilon_2$, and it has binding potency or no binding potency. We characterize every nanoparticle cluster by parameter $N_{mk}$, with the $m$-index referring to the cluster multiplicity and the $k$-index ($1 \leq k \leq m$) referring to the number of nanoparticles in the cluster that have
Table S1) Matrix of single nanoparticles forming doublets. Parameters $N_{mk}$ are indicated. Single nanoparticles have binding potency ($N_{11}$) or no binding potency ($N_{10}$). Single nanoparticles target epitope $\varepsilon_1$ or epitope $\varepsilon_2$. Doublets $N_{21}$ are formed of one nanoparticle with and one nanoparticle without binding potency. Doublets $N_{22}$ are formed of two nanoparticles that both have binding potency.

binding potency. After the incubation phase but before magnetic pulsation (so when inter-nanoparticle bonds are not yet formed) the number of single nanoparticles with binding potency is given by $N_{11}^{\varepsilon_1} = N_{11}^{\varepsilon_2} = (N/2) \cdot P_1(\lambda)$ and the number of single nanoparticles without binding potency equals $N_{10}^{\varepsilon_1} = N_{10}^{\varepsilon_2} = (N/2) \cdot \left[1 - P_1(\lambda)\right]$. Now we consider the regime of low biomarker concentration where $\lambda = C_b / C_N << 1$, which is the regime in which only doublets are statistically likely to form. Table 1 shows a matrix for doublet formation, with on the axes the species of single nanoparticles and inside the matrix the doublet species that can be formed by biomarker-induced inter-particle binding. Two types of doublets can be formed: doublets $N_{21}$ in which only one nanoparticle has binding potency (on the diagonal) and doublets $N_{22}$ in which both nanoparticles have binding potency (off the diagonal).

When all nanoparticles with binding potency have indeed formed doublets (so when $N_{11} = 0$) then the population of single particles is given by $N_1 = N_{10} = N_{10}^{\varepsilon_1} = N_{10}^{\varepsilon_2}$, and the population of doublets is given by $N_2 = N_{21} + N_{22}$. The conservation of nanoparticles in the assay tells us that $N = N_1 + 2N_2$. The conservation of binding potency gives $N \cdot P_1(\lambda) = N_{21} + 2N_{22}$. Furthermore, the ratio between the two doublet species is given by $N_{21}/N_{22} = \left[1 - P_1(\lambda)\right]/P_1(\lambda)$. By combining these
equations we find that the total number of biomarker-induced doublets equals
\( N_2 = N \cdot P_1(x)/[1 + P_1(x)] \). We can now write an expression for the total
optical scattering signal generated by doublets in solution:

\[
S(x) = \sum N_x N_x \xi \geq S_2 \left[ N_2'' + \left( N - 2N_2'' \right) \frac{1 - e^{-\xi x \Gamma}}{2 - e^{-\xi x \Gamma}} \right]
\]  
(S.5)

Here \( S_2 \) is the signal per doublet, and \( N_2'' \) is the background level of non-
specific doublets resulting from the particle fabrication process and not caused
by biomarkers. Equation (S.5) is used to fit the doublet regime of the dose-
response curves in Fig. 3, where the measured critical frequency (Fig. 3a)
identifies the regime where doublets are the dominant cluster species.
When the product \( \xi x \Gamma \) approaches unity, then most nanoparticles will have
binding potency and clusters of second order (doublets) and higher order
(multplets) will appear in solution. These species are of uniaxial symmetry and
are thus oriented in a magnetic field. As a result, the process to grow doublets
into multplets is sterically hindered, because only biomarkers captured at the
extremities can generate further cluster elongation (see inset of Fig. 3b). We
have verified by video-microscopy that indeed only linear clusters are
generated in our experiments. We describe the effective binding areas at the
cluster extremities as spherical caps with effective height \( h \) (\( 0 \leq h \leq R \), with \( R \) the
nanoparticle radius). For a cluster with multiplicity \( m \), the ratio between the
area of the two spherical caps and the total cluster surface area is given by the
area ratio parameter \( \rho = h/mR \). A full calculation of the cluster evolution in the
multplet regime is outside the scope of this paper. As a first approximation,
we assume a system that initially consists of doublets and that transforms into
quadruplets. The probability \( P_2 \) that a doublet has binding potency is given by
the chance that a biomarker is captured at a cluster extremity:

\[
P_2(x) = p(b \geq 1, \rho \xi x \Gamma) = 1 - e^{-\rho \xi x \Gamma}
\]  
(S.6)

The total number of nanoparticles in the system is constant, therefore
\( N = 2N_2 + 4N_4 \). The process of inter-doublet binding reduces the number of
doublets to \( N_2 = N/2 \cdot \left[ 1 - P_2(x) \right]/\left[ 1 + P_2(x) \right] \) and increases the number of
quadruplets to \( N_4 = N/2 \cdot P_2(x)/\left[1 + P_2(x)\right] \). Consequently, the optical scattering signal can be described as:

\[
S(x) \equiv S_2 N_2 + S_4 N_4 = \frac{N}{2} \frac{S_2 e^{-\rho \xi \xi \Gamma} + S_4 \left(1 - e^{-\rho \xi \xi \Gamma}\right)}{2 - e^{-\rho \xi \xi \Gamma}} \quad (S.7)
\]

A fit of equation (S.5) to the doublet regime of the buffer dose-response curve with linker surface chemistry (Fig. 3b) gives values for the signal per doublet (\( S_2 = 0.29 \pm 0.11 \text{ V/} \sqrt{\text{Hz}} \)), the number of non-specific doublets (\( N_2^\text{ns} = 212 \pm 73 \)) and for the steric availability \( \xi = 0.43 \pm 0.12 \). In the fit we used \( N=1943 \pm 253 \) for the total number of nanoparticles in the optical detection volume during our experiments. The fitted value for the blank signal is \( 62 \pm 45 \text{ V/} \sqrt{\text{Hz}} \), in agreement with the experimental value of \( 70.1 \pm 4.7 \text{ V/} \sqrt{\text{Hz}} \). A fit of equation (S.7) to the multiplet regime of the buffer dose-response curve (Fig. 3b) gives values for the signal per doublet (\( S_2 = 0.22 \pm 0.06 \text{ V/} \sqrt{\text{Hz}} \)), the signal per four-particle cluster (\( S_4 = 2.72 \pm 0.43 \text{ V/} \sqrt{\text{Hz}} \)) and for the area ratio (\( \rho = 0.013 \pm 0.005 \)). From the area ratio we derive a height of the spherical binding caps of \( h = 13 \pm 5 \text{nm} \).

A fit of equation (S5) to the doublet regime of the dose-response curve in undiluted plasma (Fig. 3c) gives a value for the signal per doublet (\( S_2 = 0.31 \pm 0.10 \text{ V/} \sqrt{\text{Hz}} \)), the number of non-specific doublets (\( N_2^\text{ns} = 545 \pm 118 \)) and for the Langmuir absorption factor (\( \Gamma = 0.72 \pm 0.25 \)), assuming a steric availability factor \( \xi = 0.43 \) (see above). The fit gives a blank level of \( 168 \pm 92 \text{ V/} \sqrt{\text{Hz}} \), in agreement with the measured value of \( 125 \pm 26 \text{ V/} \sqrt{\text{Hz}} \). The fit in the multiplet regime gives a value for the signal per doublet (\( S_2 = 0.20 \pm 0.07 \text{ V/} \sqrt{\text{Hz}} \)), the signal per four-particle cluster (\( S_4 = 2.18 \pm 0.39 \text{ V/} \sqrt{\text{Hz}} \)) and the area ratio (\( \rho = 0.010 \pm 0.006 \)). The area ratio yields a height of the spherical binding caps of \( h = 10 \pm 6 \text{nm} \).

Compared to the dose-response curve with linker surface chemistry, the dose-response curve with standard surface chemistry (Fig. 2c) is shifted to higher concentrations by about a factor 2.5. This means that the product \( \xi \Gamma \) is a factor 2.5 lower, i.e. \( 0.16 \pm 0.04 \). The value of \( \Gamma \) can be estimated as follows. The data in Figs. 2b and 3b show that the time constant for inter-nanoparticle
binding is a factor three larger for the standard surface chemistry than for the linker surface architecture. If we assume that the same ratio occurs during the capture phase, we obtain $\Gamma = 0.63 \pm 0.08$. Consequently, we estimate that $\Xi = 0.26 \pm 0.07$ for the standard surface chemistry.
References

In Chapter 3, the theory for rotating two-particle clusters, based on the microscopic properties of the nanoparticles, was described and experimentally tested. When performing an assay over several orders of magnitude in biomarker concentration, clusters of more than two particles appear, as shown in Chapters 4 and 5. In this Chapter, we present a model for cluster of arbitrary length and compare it with a preliminary set of experiments.
6.1 – Introduction

The controlled rotation of two-particle clusters, exhaustively studied in the previous chapters, paved the way for the opto-magnetic biosensor discussed in chapter 4. The rotational dynamics of individual clusters has been first theoretically addressed and experimentally confirmed, thereafter this understanding has been harnessed to interpret the optical signal measured when probing ensembles of doublets. Deviations from the calculated signal of two-particle clusters have been attributed to the formation of multi-particle clusters, however up to now the correlation has been only qualitative.

Aim of this chapter is to provide a theoretical framework to describe rotation of chains of arbitrary length and characterize experimentally their rotational dynamics and optical response to a wavelength and polarization.

6.2 – Model and comparison with experiments

We consider the case of a linear cluster of \( m \) superparamagnetic particles floating in a viscous liquid in the presence of an external magnetic field \( \vec{H} \), rotating in the \( xy \) plane with angular velocity \( \omega_f \). Each particle can be described by a magnetic moment \( \vec{m} = \vec{m}^{(i)} + \vec{m}^{(p)} \), where \( \vec{m}(p) \) is the permanent moment and, to first order in the field, the induced magnetic moment of the particle is given by \( \vec{m}^{(i)} = \chi V \vec{H}_{\text{ext}} \), with \( \chi \) the susceptibility of the particle material and \( V \) the volume of the particle. We assume that \( \vec{m}^{(p)} \) is constant for small fields, that is, no remagnetization occurs. This linear approximation is valid for \( H_{\text{ext}} \ll H_{\text{sat}} \), where \( H_{\text{sat}} \) is the field that saturates the magnetic moment of particle. In time varying fields, the non-zero relaxation time of the grains can cause the induced magnetization to have a time delay with respect to the field. The time delay can be accounted for by a complex frequency-dependent magnetic susceptibility \( \chi \) (see chapter 3).

In the following we will derive the equations of motions for a cluster of multiplicity \( m \) in an applied rotating magnetic field. The equations are based on the magnetic properties of the particles (the induced and permanent magnetic moments of each particle and the magnetic coupling between the particles) and the viscous properties of the fluid medium. We will derive the equations by calculating the magnetic torque generated by the cluster in the applied rotating field.
First we calculate the magnetic energy, which allows us to calculate the magnetic torques. In a dipole approximation, the magnetic energy of a cluster in the external field is the sum of two contributions, namely of the field interacting with individual dipoles and each dipole interacting with the others. Due to the rapid decay of the dipole field \((r^{-3})\), we only consider first neighbor dipole interactions. Additionally, we introduce the approximation that the particles have the same magnetic content and the same radius. We approach the solution of the problem in a recursive way, similarly to chapter 3. We first assume that the field induces the magnetic moment in the same direction for each nanoparticle, thereafter we compute the influence of the dipole-dipole interaction as second-order perturbative effect. An expression for the magnetic energy can be written both for induced and permanent moment, similarly to the derivation of chapter 3:

\[
U_i = -\mu_0 \sum_{k=1}^{m} m^{(i)}_k H_{\text{ext}} \cos(\varphi_f - \varphi_i) + \\
- \sum_{k=1}^{m-1} \frac{\mu_0}{4\pi (2R)^3} m^{(i)}_k m^{(i)}_{k+1} [3 \cos^2(\varphi_i - \varphi_k) - 1] 
\]

(6.1)

\[
U_p = -\mu_0 m_p H_{\text{ext}} \cos(\varphi_f - \varphi_p), \text{ with } m_p = \sum_{k=1}^{m} m^{(p)}_k 
\]

(6.2)

where we neglected the term describing the interaction between the two permanent moments, due to their small magnitude. In equation (6.1) \(R\) is the radius of the particles, \(\mu_0\) is the permeability of the vacuum, \(\varphi_f\) represents the angle between the horizontal \(x\)-axis and the rotating field, \(\varphi_i\) the direction between the \(x\)-axis and the induced moment of each nanoparticle and \(\varphi_k\) is the angle between the \(x\)-axis and the segment connecting the centers of the \(k\) and \(k+1\) nanoparticle. During rotation a chain of \(m\) particles can bend to minimize the viscous drag during rotation, which results in a segmented shape for the chain. In our biosensor, the multiplicity of the chains is limited by the Mason number during the actuation phase and by the available surface for binding (see chapter 5). Consequently, we can safely neglect the influence of buckling and assume \(\varphi_k \approx \varphi_c\). In equation (6.2) \(\varphi_p\) is the angle between the vector sum of all the permanent moments and the \(x\)-axis. Evaluating the sums, equations (6.1) and (6.2) can be simplified to:

\[
U_i = -m \frac{4}{3} \pi R^3 \chi \mu_0 H_{\text{ext}}^2 \cos(\varphi_f - \varphi_i) + \\
- (m - 1) \frac{\mu_0 \chi^2 H_{\text{ext}}^2 R^3}{18} [3 \cos^2(\varphi_i - \varphi_c) - 1] 
\]

(6.3)
The configuration of minimum energy corresponds to the situation in which the induced moments are all aligned with the field and the axis of the particle cluster. Any deviation from this configuration leads to an increase in the magnetic energy and a corresponding torque acting on the chain:

\[ \tau_i = \frac{\partial U_i}{\partial (\phi_i - \phi_c)} + \frac{\partial U_i}{\partial (\phi_f - \phi_i)} = \mu_0 n H_{ext}^2 \chi R^3 \times \]

\[ \times \left\{ \frac{(m-1)}{6} \chi \sin[2(\phi_i - \phi_c)] + \frac{4m}{3} \sin(\phi_f - \phi_i) \right\} \tag{6.4} \]

\[ \tau_p = \frac{\partial U_i}{\partial (\phi_f - \phi_p)} = \mu_0 m_p H_{ext} \sin(\phi_f - \phi_p) \tag{6.5} \]

From a closer inspection of equation (6.4), the induced moment contributes in two distinct ways to the magnetic torque, namely by dipole-dipole interaction and by the lag between field and induced moments. Being a first order effect, the angular difference between field and induced moment is expected to be extremely small. The physical reason lies in the rapid Néel relaxation time, which results in the induced moment to be in equilibrium with the external field up to \( \omega_f \sim \text{MHz} \). We now derive the equation of motion for low frequency \((\omega_f \ll \tau_{rel})\), with \( \tau_{rel} \) the relaxation time of the grains of the superparamagnetic particle. Consequently, we will neglect the contribution to the induced torque deriving from the lag between field and induced moments, which result in a total magnetic torque:

\[ \tau_m = \mu_0 H_{ext} \left\{ \frac{(m-1)H_{ext} \pi \chi^2 R^3}{6} \sin[2(\phi_i - \phi_c)] + m_p \sin(\phi_f - \phi_p) \right\} \tag{6.6} \]

We now proceed to evaluate the hydrodynamic torque. We consider a chain of \( m \) particles rotating around its center of mass and, as a first approximation, we neglect the weak hydrodynamic coupling between the particles. The motion can be decomposed into two orthogonal components, that is, a rotation around the center of each particle with angular velocity \( \omega_c \) and a translation about the axis with velocity \( \vec{v} = \vec{\omega}_c \times \vec{r}_k \), where \( \vec{r}_k \) is the distance between the center of the \( k \) particle and the origin. The rotational component of the motion is independent of the position in the chain and each particle contributes to the torque with an amount \( \tau_r = 8\pi \eta R^3 \omega_c \), where \( \eta \) is the viscosity of the fluid medium. The vector \( \vec{r}_k \) has magnitude:
\[ r_k = 2R \left[ 2k - \text{mod}(m, 2) \right] \]  \hspace{1cm} (6.7)

where \( \text{mod}(x, y) \) is a function that returns the rest after division of \( x/y \). The total hydrodynamic torque acting on a chain is then given by:

\[
\tau_{\text{hydro}} = \sum_{k=1}^{m} \tau_r + \sum_{k=1}^{m} \vec{F}_k \times \vec{r}_k = m(8\pi \eta R^3 \omega_c) + \sum_{k=1}^{m} 6\pi \eta R \vec{v} \times \vec{r}_k, \hspace{1cm} (6.8)
\]

where \( \vec{F}_k \) is the friction force acting on an isolated sphere. It can be demonstrated that the sum in (6.8) is independent of the parity of the chains. The physical rationale lies in the fact that in case of an odd number of particles in the chain \( r_1 = 0 \) for the central particle, resulting in exactly the same torque as in the case of even \( m \). By computing (6.8), we obtain the dependency of the hydrodynamic torque on the chain length:

\[
\vec{\tau}_{\text{hydro}} = 8\pi \eta R^3 \frac{m^3 + 3m}{4} \vec{\omega}_c \hspace{1cm} (6.9)
\]

Equation (6.9) has been derived under the approximation of neglecting the hydrodynamic coupling between the particles. An expression for the viscous drag experienced by a chain of \( m \) spheres rotating around the center of mass including hydrodynamic coupling has been previously derived:

\[
\vec{\tau}_{\text{hydro}} = 8\pi \eta R^3 \frac{m^3}{3 \ln(m/2)} \vec{\omega}_c \hspace{1cm} (6.10)
\]

Fig. 6.1) Theoretical prediction for the hydrodynamic torque (with and without hydrodynamic coupling) as a function of the chain length for 500nm nanoparticles.
Equation (6.10) is valid only for $m > 3$ and therefore the hydrodynamic coupling has been neglected in the development of equations for two-particle clusters. In Fig. 1 we compare equations (6.9) and (6.10) as function of $m$, where the values for (6.10) corresponding to $m=2$ and $m = 3$ have been extrapolated by fitting the curve (imposing that for $N=1$ the hydrodynamic torque needs to be equal to $8\pi \eta R^3 \omega_c$).

To get to the equation of motion for both the external dynamics (described by the axis of symmetry of the cluster) and for the internal dynamics (described by the orientation of the induced moment), we first note that the Reynolds number, given by the ratio between inertial and viscous forces, is much smaller than unity. Consequently, inertial terms can be neglected and the motion of the chain is described by balancing magnetic and hydrodynamic torque:

$$\frac{d\varphi_c}{dt} = \omega_{\text{crit}}^{(i)} \sin[2(\varphi_i - \varphi_c)] + \omega_{\text{crit}}^{(p)} \sin(\varphi_f - \varphi_p)$$  \hspace{1cm} (6.11)

where

$$\omega_{\text{crit}}^{(i)} = \frac{m-1}{m^3} \ln \left( \frac{m}{2} \right) \frac{\mu_0 x^2 H_{\text{ext}}^2}{16 \eta}, \quad \omega_{\text{crit}}^{(p)} = \frac{3}{m^3} \ln \left( \frac{m}{2} \right) \frac{m^2 \mu_0 H_{\text{ext}}}{8 \pi \eta R^3}$$  \hspace{1cm} (6.12)

In equation (6.12) the parameter $\omega_{\text{crit}}^{(i)}$ and $\omega_{\text{crit}}^{(p)}$ represent the critical frequencies respectively for the induced and the permanent moment. In chapter 3 we demonstrated that the most reproducible regime for the application of the torque is when the induced moment is dominant over the permanent moment ($B \gtrsim 1 \text{mT}$). In this regime, the parameter $\omega_{\text{crit}}^{(i)}$ represents the critical frequency. The right panel of figure 2 shows the dependence of the critical frequency as a function of number of particles in the chain.

![Fig. 6.2](Left panel) Critical frequency as a function of number of particles in a chain, for $\chi=2.5$, $B=4 \text{mT}$, $\eta=0.0025 \text{ Pa}\cdot\text{s}$. (Right panel) Calculated phase lag between applied field and induced moments at the critical frequency as a function of chain length for a susceptibility of $\chi=2.5$. 

![Graphs](Left panel) Critical frequency as a function of number of particles in a chain, for $\chi=2.5$, $B=4 \text{mT}$, $\eta=0.0025 \text{ Pa}\cdot\text{s}$. (Right panel) Calculated phase lag between applied field and induced moments at the critical frequency as a function of chain length for a susceptibility of $\chi=2.5$. 

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The internal dynamics can be calculated by noting that the magnetic moments relax extremely rapidly; consequently the orientation of the induced moment can be considered in equilibrium with the external field at any instant in time. The magnetic energy is in a local minimum, which can be computed to evaluate the relative orientation between induced moments and external field:

\[
\frac{\partial u}{\partial \varphi_i} = 0 = \sin(\varphi_i - \varphi_f) + \chi^{m-1}_{8m} \sin[2(\varphi_i - \varphi_c)]
\]  

Equation (6.13) allows for an estimation of the maximum lag between field and induced moments as a function of chain length (see Fig. 2). Such lag is maximum at the critical frequency, when all the magnetic torque available is spent to overcome the viscous drag. The calculated values show that the lag rapidly increases with the chain length and reaches a plateau at \(\sin^{-1}(\chi/8)\). The calculated phase lag is consistent with the previously found value (3° to 10°) for a doublet composed of 1μm particles. The equilibrium orientation of the induced moments derives from the balance between field-dipole and dipole-dipole interactions. For increasing chain lengths, the dipole-dipole interaction becomes more and more predominant and the moments align energetically more favorable to the axis of the chain than to the external field.

At high frequencies \((\omega_f \sim \tau_{rel}^{-1})\), the magnetization of each grain starts lagging behind the external field. Consequently, the constraint (6.13) is no longer valid and the quantity \(\varphi_f - \varphi_i\) becomes not negligible, generating a contribution to the torque (see equation (6.4)). All the other terms in equation (6.4) vanish and the equation of motion simplifies to:

\[
\frac{d\varphi_c}{dt} = \frac{\ln(m/2) \mu_0 H_{ext}^2}{m^2 2\eta} \chi''
\]

where we note that the quantity \(\sin(\varphi_f - \varphi_i)\) equals the complex part of the magnetic susceptibility.

The derivation of the equations of motion based on the balance between magnetic torque and hydrodynamic torque provides a theoretical framework to understand the dose-response curves of chapters 5 and 6. We first compute the expected values for the critical frequency according to (6.12) and we compare the theoretical value with the critical frequency experimentally measured. Given that the sample is characterized by a widely distributed cluster size, we assume that the critical frequency corresponds to the most abundant cluster species, by rounding each experimental point to the closest theoretical
Fig. 6.3) Panel a shows the measured critical frequency as a function of target concentration. The data show a constant value for the critical frequency at low concentrations, which defines the doublet regime (see chapter 5). The two assays studied, bBSA and PSA, show an overlap in the values of critical frequency, which suggests that the cluster species formed are in majority identical for a given target concentration. This is shown in panel b, where the cluster size, extrapolated by the value of critical frequency according to (6.12), is plotted as a function of target concentration. The theoretical values used are $\chi=2.5$, $B=3.5$ mT, $\eta=0.0025$ Pa·s. In the estimation of the hydrodynamic torque, the value including the hydrodynamic coupling has been used. For $m=2$ and $m=3$ the value has been extrapolated by fitting the calculated points for $m>3$ and by imposing that the torque for $m=1$ coincides with an isolated sphere. Remarkably, it appears that clusters with even multiplicity are preferably formed. After depletion of single particles, doublets assemble into clusters with $m=4, 6, 8, \ldots$. Panel c shows the measured critical frequency as a function of the number of estimated particles in the sample. The decreasing trend is in good agreement with formula (6.12).
explanation is that the population of clusters transits from doublets to triplets and four-particle clusters by depleting single particles from the solution. At higher concentrations, the formed clusters assemble by mutual binding to form high multiplicity clusters, however, having the single particles being depleted, only even multiplicity can occur. The multiplicity of the chains as function of biomarker concentration presents a step-like behavior, where the width of the steps decreases progressively. This behavior is consistent with the decreased binding area available for progressively longer chains, as described in chapter 5. The doublet and triplet regimes extend over a wide concentration range, corresponding to progressive depletion of single particles. Before chains of multiplicity larger than 3 can be formed, accessible biomarkers need to be captured onto the extremities of doublets and triplets (because of the constraint of uniaxial clusters). The probability of such process occurring scales with the ratio between accessible and total area and is proportional to the inverse of the multiplicity of the chains. Consequently, we observe a threshold-like behavior, where depletion of single particles imposes a hard constraint that demands considerable biomarker concentrations before high-multiplicity clusters appear. Remarkably, no significant difference is visible between the two assays. This suggests that the statistical distribution of the population of cluster sizes is identical, regardless of the particular biomarker. The magnetic actuation differs considerably between the two assays, however in both cases the actuation is applied long enough to reach saturation for each concentration point. Consequently, it is likely that the amount of binding is determined by steric factors.

Figure 6.3c reports the measured critical frequencies as a function of estimated number of particles for two targets (bBSA and PSA) concentration. The data are consistent with the theoretical predictions, however an experimental verification of the size distribution of the cluster is desirable. We therefore envision as future work the analysis real-time by video microscopy of the sample composition and its correlation with the critical frequency measured by means of optical scattering.

6.3 – Conclusions

In conclusion, we presented a theoretical model for a uniaxial chain of magnetic particles of arbitrary multiplicity, rotating in an external magnetic field. The torque applied scales with the multiplicity of the clusters and it can
be accurately controlled in fluid. With the support of the theoretical model we can infer the evolution of the size of the cluster population while performing immunoassays. The found values are in consistency both with the experimentally measured critical frequencies and the theoretical model presented in chapter 5.

References

Conclusion & Perspective
In this dissertation, we have presented a novel biosensing technology based on detection in solution of rotating clusters of magnetic nanoparticles. Chapter 3 describes a study of the theory of rotation of two-particle clusters. The induced and permanent moments of the nanoparticles with the external field and generate a torque which counteracts the viscous drag due to the motion of the clusters. A comparison between theory and experiments results in a precise characterization of the permanent moment (mp) and susceptibility (χ) of individual clusters. For the 1 μm particles used in our experiments, we measured χ=2.65 and mp=1.5×10⁻¹⁶ A m². Knowing the magnetic properties of the particles, the viscosity of biological samples can be determined. We found that the viscous drag experienced by a two-particle cluster rotating in human plasma can be quantified and thereby the viscosity of the sample. In chapter 4 we studied the feasibility of detecting ensembles of clusters in solution. By rotating the clusters, a temporal modulation of the scattered light is generated because of their uniaxial symmetry. Single nanoparticles are spherically symmetric, thus they do not modulate the incoming light. The optical signal, analyzed in the frequency domain, reveals the number, size and magnetic properties of the clusters. We studied a model two-step assay and demonstrated a 5 pM limit of detection of biotinylated-BSA in human plasma in less than 3 minutes. In chapter 5, we studied the possibility of implementing a one-step homogeneous immunoassay directly in blood plasma, avoiding fluid manipulations. We first designed a magnetic actuation protocol based on pulses of rotating fields. Pulsation increments the number of collisions between different nanoparticles and randomizes the relative angular orientation by enabling Brownian rotation and diffusion. Compared with continuous actuation, pulsation gives two times a higher rate of cluster formation. Modifications of the surface chemistry of the nanoparticles enables a one-step immunoassay by reducing the non-specific interactions more than an order of magnitude. With this approach, we demonstrated a limit of detection of 500 fM in blood plasma in less than 15 minutes. Chapter 6 extends the theory of rotation to a chain of arbitrary number of particles. The measured critical frequency can be correlated to the size of the most abundant cluster species in the assay, providing a monitoring tool for the immunoassay. The critical frequency is highest for two-particle clusters and decreases for increasing number of particles in the cluster, with a power law with exponent close to 0.8. The phase lag between the magnetic field and the axis of symmetry of the
cluster is comprised between $3^\circ$ and $10^\circ$ for small number of particles, and it asymptotically increases to a value close to $18^\circ$ for long chains.

We envision that this versatile nanotechnology will enable several fundamental studies in optics and biophysics. Our technique enables studies of the angle-dependent optical scattering properties of clusters of superparamagnetic nanoparticles. The influence of optical parameters such as wavelength and polarization could be evaluated over the full $4\pi$ solid angle. The high sensitivity with respect to the early stages of particle aggregation may be used to monitor the monodispersity of batches of nanoparticles and their uniformity in terms of size and magnetic properties. Different bioactive coatings could be tested to assess their effectiveness for capturing target molecules or enhancing the colloidal stability. Metallic coatings may enhance the modulation signal, thus achieving better sensitivity. Particle sizes, particle concentrations and magnetic fields may be varied in order to enable even more rapid assays. Furthermore the feasibility of different biological assays (e.g. nucleic acids, small molecules) can be investigated. We believe that our nanobiotechnology will pave the way for novel fundamental and applied studies, offering interesting challenges for the years to come.
Curriculum Vitae

Andrea Ranzoni was born on 3rd January 1983 in Voghera, Italy. After finishing his Science Diploma in 2000 at Liceo Scientifico Galileo Galilei in Voghera, Italy, he studied Physical Engineering at Politecnico di Milano, where he obtained his Bachelor and Master of Science degrees. He performed his graduation project in 2007 at the National Research Center of Turin, working towards the implementation of hyper-entangled bi-photons for quantum cryptography applications. In the same year he was awarded the Diploma of Alta Scuola Politecnica and in 2008 he obtained a Master of Science in Electronic Engineering from the Politecnico di Torino, Italy. From September 2007 he moved to the Netherlands for his PhD research, which he performed at Philips Research Laboratories in collaboration with Eindhoven University of Technology.
List of Publications

Papers:


Patents:

Acknowledgements

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accompanied my time over the first few years. Crystal taught me how versatile pink can be, Christiane showed us all that a sparkling character goes well with fizzy shots, Emilie knows how hard my head can be, Francesco for teaching me how to travel posh and how to run after a camel ride, thanks to him my ample common sense was often questioned. Aude my messy housemate, who showed me the wonders of macro-photography. We had long and nice chats, few lovely sushi experiences and very nice home-made dinners. Fiona, another sparkling character that wanted me as private chef and who taught me that falling from a horse can be painful. Paul and Simon, the sunflowers who designed the best table-football ever made on the planet. Thanks for the lovely time together, we left Eindhoven more or less at the same time and I wish you all the best. A massive thanks to Paul, who kindly designed the cover of this thesis. Lovely people I also met here in Philips, Ula and Tomek who will always remember my Christmas dinner while I will remember how dogs can become fairly mad, Thiru who gave me the chance of experiencing a real Indian wedding, showering with forty of fever under an icy waterfall, climbing coconuts and contemplating the colors, warmth and peace of an awesome beautiful country. Jessica, who welcomed me here giving me a place to stay when I knew nobody and who initiated me to my corporate life. Of course Ruben, who taught me that an Indian can make the best arancini ever, who showed me how Indians can party with people on the other side of the planet, that joined me in the fun of volleyball and countless little things of which I am grateful. I wish him and the School Teacher all the best! My fellow Kim, who shared the path of PhD in Philips with me for most of my time, I really wish her happiness in the new challenges she has chosen. Markus and Ke, we started together and many things happened in between, mostly two kids and a Polish lady. Raffa and Gatto who taught me that the smell of sage is different on each side. The patient Gwen of whom I enjoyed a lot the company and who enjoyed a lot my cooking workshops, especially the seven chilos of gnocchi, eh Remco?! Eva and Leonie, thanks for the nice chats while sharing the best office ever. Kalyan and Jyostna, for great meals, nice biking trips and nice travelling together. Getting lost in The Netherlands might be hard but we always managed to. Gregor, for the witty chess games and lovely banter, the posh bars and the wild parties. A ceramic cup filled with triples often replaced the good will of long runs in the countryside with your cinema trousers. We’ll always remember the trip
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