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„Synthesis and surface chemistry of nickel nanoparticles for protein biomarker-based molecular diagnostics“

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Publications

- Schrittwieser, S., Reichinger, D., Schotter, J.; Applications, Surface Modification and Functionalization of Nickel Nanorods. *Materials* **2018**, *11*, 45; doi:10.3390/ma11010045

Abstract

Molecular diagnostics relies on the detection of biomarkers in complex sample solutions. Of special relevance are point-of-care testing methods which circumvent the need for laborious sample preparation and sophisticated instrumentation. One representative is the PlasMag measurement principle which relies on biofunctionalized magnetic nanorods and changes of their physical properties upon biomarker binding to their surface. This thesis focuses on the electrochemical synthesis and modifications of the surface chemistry of nickel nanorods. The aim was to fabricate stable dispersions of nanorods that comprise a functional surface that paves the way for future biosensing experiments. The synthesis of nickel nanorods in colloidal suspension is a three-step process. First, nano-sized porous aluminium oxide layers were produced using an anodizing process in acidic solution. Second, the resulting pores are filled with nickel from a solution of nickel salts *via* pulsed electrodeposition. In a final step, the nanorods were released from the aluminium oxide template and dispersed in an appropriate solvent after several washing steps. The nanorods possessed a length of about 200 nm and a diameter of approximately 15 nm as determined by scanning electron microscopy. By stabilizing the so obtained dispersions, nanoparticle agglomeration effects due to attractive van der Waal's forces and magnetic dipole-dipole forces can be compensated. This can be achieved by a suitable modification of the nanorod surface chemistry with either small molecules or polymers. For this reason, stabilization approaches by electrostatic, steric or electrosteric repulsion were employed to modify the nanorod surface. An example for a polymer that allows for electrosteric nanorod dispersion stabilization is the water-soluble polymer PVP that adsorbs to the surface of the nickel nanorods through its hydrophilic pyrrolidone group. Due to the resulting polymer layer with varying chain lengths, steric repulsions prevented particle agglomeration. Stability of the nanorod dispersion was confirmed by zeta potential measurements which indicated, that electrosteric repulsions between the coated particles prevent agglomeration. Another possible approach for particle dispersion stabilization is to use the high affinity of carboxylic acids to the native nickel oxide layer which provides theoretical basis for stabilization approaches with fatty acids like oleic acid and lauric acid. To that end, the particles were coated with fatty acids to produce a hydrophobic layer, which was then further used as a priming layer for the coating with an amphiphilic polymer. Here, the highly negative zeta potential indicated high stability of the aqueous nanoparticle dispersion, which allows for future antibody conjugation. The short-term stability of particles with different coatings was determined using the PlasMag measurement method. This successful nanorod synthesis and surface modification offers the possibility for a future conjugation of the nanorod surface by the monoclonal antibody trastuzumab to enable for a benchmarking of the PlasMag system by the detection of the sHER2 biomarker.

Zusammenfassung

Ziel der Masterarbeit war es, zylindrische Nickel-Nanopartikel auf elektrochemischem Weg zu synthetisieren und diese in wässriger Lösung zu stabilisieren. Zudem sollte die veränderte Oberflächenchemie der Nanopartikel die Konjugation von Antikörpern im nächsten Schritt erlauben und so den Einsatz der biofunktionalisierten Nanostäbchen zur Proteindetektion mit der PlasMag Messmethode im Rahmen der patientennahen, einfach handhabbaren Labordiagnostik, sowohl in Pufferlösungen als auch in Serums- und Speichelproben ermöglichen.

Die Herstellung der Nanopartikel erfolgt über ein template-basiertes Verfahren, in welchem zuerst die Oberfläche einer Aluminiumfolie in einer sauren Lösung oxidiert wird und anschließend elektrochemisch ein Porenwachstum in der Oxidschicht induziert wird. Diese Poren werden in einem weiteren elektrochemischen Syntheseschritt mit Nickel befüllt, wodurch zylindrische Nickel-Nanopartikel mit einer Länge von ca. 200 nm und einem Durchmesser von ca. 15 nm erhalten werden, wie mittels Elektronenmikroskopie bestimmt wurde. Im nächsten Arbeitsschritt wurde die Oxidschicht nass-chemisch aufgelöst und damit die Nanopartikel in Lösung gebracht. Gleichzeitig wurde dafür Sorge getragen, dass die entstandene Nanopartikeldispersion stabil ist, bzw. die Agglomeration der Nanopartikel verhindert wird, indem anziehende Van der Waals Kräfte und magnetische Dipol-Dipol Kräfte durch sterische, elektrostatische oder elektrosterische Wechselwirkungen kompensiert werden. Die Modifikation der Oberfläche erfolgte auch in Hinblick auf eine weitere Funktionalisierung der Nanopartikel mit Antikörpern. Dies bedeutet, dass die eingesetzten Surfactants reaktive funktionelle Gruppen wie z.B. Carboxy Gruppen aufwiesen, um in einem zukünftigen Schritt die kovalente Anbindung eines Antikörpers zu ermöglichen.

Das wasserlösliche Polymer PVP ist ein Beispiel für ein oberflächenaktives Molekül, welches durch elektrosterische Wechselwirkungen die Nanorod-Dispersion stabilisieren konnte. Es adsorbiert mit der hydrophilen Pyrrolidon-Funktionalität an die Oberfläche der Nickel-Nanopartikel und stabilisiert diese durch die unterschiedlichen langen Polymerketten, die sterische Abstoßungen zwischen den beschichteten Partikeln verursachen. Die moderate Stabilität dieser Dispersion wurde durch Messungen des Zeta-Potentials bestätigt, welche auch die elektrostatischen Abstoßungen belegen konnten.

Die hohe Affinität von Carbonsäuren zu der natürlich auftretenden Nickeloxidschicht auf den Nanopartikeln bietet die theoretische Grundlage für Stabilisierungsansätze basierend auf Fettsäuren wie z.B. Ölsäure oder Laurinsäure. Durch die Adsorption der hydrophilen Carboxy-Gruppe an die Nanopartikeloberfläche ragt der hydrophobe Teil des Moleküls in die umgebende Lösung und bildet eine hydrophobe Schicht um den Partikel. Diese Ummantelung diente als Grundlage für die weitere Funktionalisierung mit dem modifiziertem, amphipathischen Polymer PMA. Das hohe, negative Zeta-Potential dieser Partikel deutete auf eine exzellente Stabilität hin, wodurch in einem zukünftigen Schritt der

monoklonale Antikörper Trastuzumab mittels EDC/sulfo-NHS Linker-Chemie an die Nanopartikel konjugiert werden kann. Zudem wurden auch Stabilitätsmessungen der unterschiedlich beschichteten Partikel in einem Zeitraum von 24 Stunden mit der PlasMag Methode durchgeführt, welche zeigten, dass die mit Ölsäure und PMA behandelten Partikel ausreichend lange stabil sind, um die Antikörper anzubinden.

Durch die erfolgreiche Synthese der Nickel Nanostäbchen und Modifizierung ihrer Oberflächenchemie konnte der Weg für eine zukünftige Konjugation des monoklonalen Antikörpers Trastuzumab geebnet werden, wodurch auch das Benchmarking des am AIT etablierten Sensorkonzepts PlasMag zur Detektion des sHER2 Biomarkers ermöglicht wird.

1 Introduction

1.1 Structure of the Thesis

Within this chapter, a general introduction to point-of-care (POC) applications and (nano)particle-based biosensing will be given and the required properties of probes for PlasMag measurements will also be elucidated. The theoretical framework of the electrochemical synthesis of nickel nanorods, their subsequent stabilization in colloidal dispersion as well as their surface functionalization will be discussed in chapter 2. Additionally, the measurement principle and basic knowledge of scanning electron microscopy (SEM) and the PlasMag setup will also be covered. In chapter 3, the experimental procedures and setups will be presented, followed by the discussion of the obtained results in chapter 4. The thesis is concluded by an outlook on future applications and possible improvements for the described procedures. The equations for the synthesis of nanoparticles are provided in the appendix.

1.2 Point-of-Care Testing

Point-of-care testing can be described as biomedical testing outside of clinical laboratories, which allows patient diagnoses in the physician's office, an ambulance, the patient's home, the field, in mobile laboratories and bed side monitoring. Instruments for POC testing have to combine multiple analytical functions into self-contained, low cost, portable devices that can be easily handled to display specific and sensitive results in a comprehensive format and, therefore detect and diagnose disease rapidly. Well-established examples of POC testing applications include blood glucose testing, blood gas and electrolytes analysis, pregnancy testing, infectious disease testing and cholesterol screening ¹.

POC testing often relies on immunoassays, which measure the concentration of a target molecule (antigen) through the use of an antibody, which is able to recognize and bind a specific molecule in a complex matrix, similar to the immune system of the human body. While heterogeneous assays have to be run in multiple steps, in which reagents are added and washed away to facilitate the identification of the target molecule through an interaction with an antibody, homogeneous assays use the "mix and measure" principle, where the reagents are simply mixed and a physical measurement is conducted. Heterogeneous measurement principles typically rely on the binding of the analyte to a two-dimensional surface in contrast to homogeneous methods, which are based on the detection of analytes throughout the entire sample volume.

The enzyme-linked immunosorbent assay (ELISA) is a popular and widely used representative of a heterogeneous assay, which is used, exemplary, as a routine diagnostic tool in medicine and toxicology ², for the detection of food allergens in the food industry ³ and for home pregnancy tests ⁴. Despite providing low background, wide dynamic range and high sensitivity ⁵, disadvantages like a high workload caused by multiple incubation and washing cycles in a laboratory setting, interfere with the requirements of POC testing applications ^{6,7}.

In contrast, the “mix and measure” principle applied in homogeneous label-free assays, reduces extensive sample preparation to a minimum, and accomplishes a fast transition to equilibrium of the analyte molecule binding process due to three-dimensional diffusion of the analyte and capture probes compared to two-dimensional diffusion in heterogeneous assays ⁸. Furthermore, it also circumvents steric hindrance of analytes binding to the flat capture surface. Taking these facts into consideration, the characteristics of homogeneous assays prove to be more suitable for POC testing applications.

1.3 Nanoparticles in Biosensing

It is possible to further subdivide homogeneous measurement methods by categorizing them into particle-free and particle-based approaches. Just like in many different technological fields, the appropriateness of nanomaterials has been proven for biosensing applications. However, the exact field of application strongly depends on the structural characteristics, especially size and size distribution, shape, potential magnetic properties and surface chemistry of nanoparticles ⁹. In general, nanoparticles are defined as particulate matter with at least one dimension that is less than 100 nm and also constitute a sub-fraction of what is defined as “colloids” by the IUPAC ^{10,11}. Several criteria including appropriate size, colloidal stability and surface functionality for conjugation of biomolecules must be provided by nanoparticles to facilitate their application in biological systems.

Biosensing devices are defined by their biological, or bioinspired receptor unit with unique specificities towards corresponding analytes and demand similar specifications as POC testing applications. The use of nano-sized objects led to lowered detection limits of several orders of magnitudes and increased sensitivities in addition to the general advantage of an enhanced surface area enabling the immobilization of an increased amount of bioreceptor units ¹².

Nanoparticles are already established as probes for homogeneous biosensors and corresponding techniques include surface-enhanced Raman spectroscopy (SERS) ^{13,14}, particle agglutination-based assays ^{15–18} and sensor principles based on magnetic particle labels ¹⁹. Magnetic nanoparticle probes offer the advantage of tunable properties through manipulation of the probes with an applied magnetic field and additionally allow frequency-selective analysis. This leads to an enhanced sensitivity due to the improved signal-to-noise ratio of the measurement signal ^{19–21}. Due to the focus on nanoparticles for

signal detection, methods involving micro- and nanoparticles for washing or separation steps are excluded from this brief introduction ²².

Signal detection strategies based on magnetic and optical detection have to be distinguished. Methods based on magnetic measurement principles either detect the presence of magnetic nanoparticles, use nuclear magnetic resonance to sense the environment surrounding the particle labels or measure changes of the hydrodynamic particle volume ^{19,23–28}. A commercial system (T2 Biosystems Inc.) relying on the nuclear magnetic resonance method has been launched, in which the clustering of magnetic nanoparticles induced by analyte molecule binding results in an altered average T_2 relaxation time of the water protons in the sample. Since all particle agglomeration effects, including ones due to unspecific binding, contribute to the measured signal, the establishment of reliable reference measurements is challenging.

The fast decay of the magnetic dipole field with distance requires the sample to be in close proximity to the detector for magnetic detection principles, while the distance between the sample and the detector does not play an important role for the optical detection. The limited flexibility of the biosensing setup design in case of magnetic detection thereby represents a clear disadvantage. Alterations of the nanoparticle orientation in the sample solution can be optically detected if the nanoparticles display optical anisotropy, which can either arise from intrinsic particle geometry and composition properties or as the result of clustering, although the latter one leads to difficulties in establishing proper reference measurements ¹⁹. So, it can be concluded, that a homogeneous assay approach relying on magnetic agitation and optical detection of dispersed nanoparticle probes with intrinsic optical anisotropy is well suitable for POC applications.

1.4 Nanoparticle Requirements for PlasMag Measurements

Homogeneous label-free immunoassays can be realized if the binding of analyte molecules to a probe results in a direct change of the measurement signal through a change of the physical properties of the probe. Measurements using the “PlasMag” principle are based on the optical observation of changes in the rotational dynamics of magnetically agitated nanorods (“nanoprobes”) upon their specific interaction with proteins and thus represent a homogeneous label-free immunoassay ²⁹.

For PlasMag measurements, nanoprobes are mixed with the sample fluid and excited by a rotating magnetic field, where they experience a hydrodynamic drag in the sample fluid and therefore follow the RMF at a phase lag angle α . The hydrodynamic shell thickens as target molecules bind to the nanoprobes and α increases. For this reason, the phase lag angle α serves as a parameter for the average number of target molecules bound to the nanoprobes. The phase lag angle α is accessible through optical measurements in transmission geometry using polarized light. The intensity of the signal depends on the nanoprobe orientation with respect to the polarization direction ³⁰. A more detailed introduction into the measurement principle can be found in section 2.5.

To manipulate the nanoparticle alignment and to measure the alignment by absorption of linearly polarized light, nanoprobe with both anisotropic optical and magnetic properties are required. Cylinder-shaped nanoparticles composed of a ferromagnetic material like nickel provide all those characteristics³⁰. As a prerequisite for the measurement principle, the nanoparticles not only have to withstand oxidation, but also must be stabilized in aqueous buffer solutions as single particle dispersions and biofunctionalization of the nanoparticle through conjugation of the respective antibody to the particle surface has to be ensured. In addition, the hydrodynamic nanoparticle size has to be close to the order of the analyte proteins to induce measurable changes in the phase lag upon analyte binding. Finally, a nanoparticle fabrication process that can be up-scaled is desired.

1.5 Molecular Breast Cancer Biomarker HER2/neu

Breast cancer is by far the most commonly diagnosed cancer among women and although earlier detection and improvements in treatments resulted in a decline in mortality rates in most EU countries, breast cancer is still the leading cause of death from cancer³¹. The causes of breast cancer are not well-understood, but age^{32,33}, family health history³⁴, major inheritance susceptibility³⁵, breast tissue density, exposure to estrogen³⁶, being overweight or obese³⁷, alcohol^{38,39}, radiation^{40,41} and hormone replacement therapy⁴² are described as risk factors.

Approximately 20-30% of breast cancer patients show an overexpression or amplification of the *ERBB2* oncogene, which encodes the receptor tyrosine-protein kinase erbB-2^{43,44}. The HER2/neu protein, as it is also called, belongs to the epidermal growth factor receptor family (HER/EGFR/ERBB) and regulates important aspects of cell growth and differentiation⁴⁵⁻⁴⁷. Since HER2 is a transmembrane protein, it consists of a transmembrane domain, an intracellular domain for the interaction with various signaling molecules and an extracellular domain which can be released from the other domains by proteolysis and shed into the blood stream resulting in soluble HER2 protein fragments, which can then be detected in serum (sHER2)⁴⁵. Amplification of the *ERBB2* gene in the amplicon of chromosome 17 causes an up to 100-fold increase in the expression level of HER2 on the surface of breast cancer cells, although the mechanisms of selective amplification remains unknown. Fig. 1 shows a healthy cell with normal levels of HER2 expression in contrast to a cancer cell with elevated levels of the HER2 protein. The most commonly used clinical cut-off level for serum sHER2 concentrations is 15 ng/ml (or approximately 143 pM with an average molecular weight of sHER2 of 105 kDa) for the US Food and Drug Administration approved ELISA⁴⁸. The ADVIA Centaur Immunoassay System by Siemens is able to measure the serum levels of HER2 using two monoclonal antibodies in a sandwich-type immunoassay and employing direct chemoluminescence for signal detection⁴⁹. Although this instrument allows access to a broad range scanning of various biomarkers for cancer, infectious disease, cardiovascular disease, etc. it is not applicable for POC testing, due to its large size.

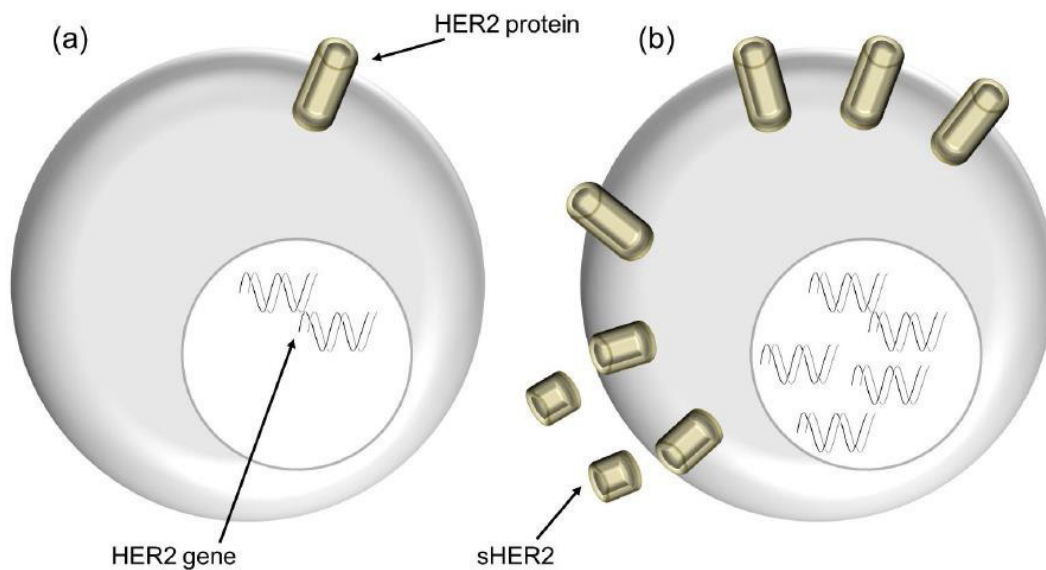


Figure 1 | HER2 expression in healthy cells (a) and in cancer cells (b). The amplification of the HER2 gene results in an overexpression of the HER2 gene in cancer cells, which ultimately leads to an increased number of HER2 receptors on the cell surface. The extracellular domain of the HER2 transmembrane protein can be shed into the blood stream by proteolysis (sHER2). Reproduced with permission from Schrittwieser, S., "Modeling and Development of a Biosensor Based on Optical Detection of the Rotational Dynamics of Hybrid Nanoparticles", Bielefeld University, AIT Austrian Institute of Technology, 2014.

A suitable antibody for targeting the breast cancer biomarker sHER2/neu is the recombinant humanized monoclonal antibody trastuzumab (marketed as Herceptin® by F. Hoffmann-La Roche AG, Switzerland). Trastuzumab is directed against the extracellular domain IV of HER2 and has cleared the way for a plethora of targeted approaches in breast cancer treatment due to the significantly improved patients' outcomes⁵⁰⁻⁵². The molecular mechanisms of action of trastuzumab can be grouped into HER2 degradation⁵³, antibody-dependent cellular cytotoxicity^{54,55} and MAPK and PI3K/Akt interference⁵⁶⁻⁵⁸ resulting in a potent suppression of cancer cell growth, proliferation and survival as can be seen in Fig. 2.

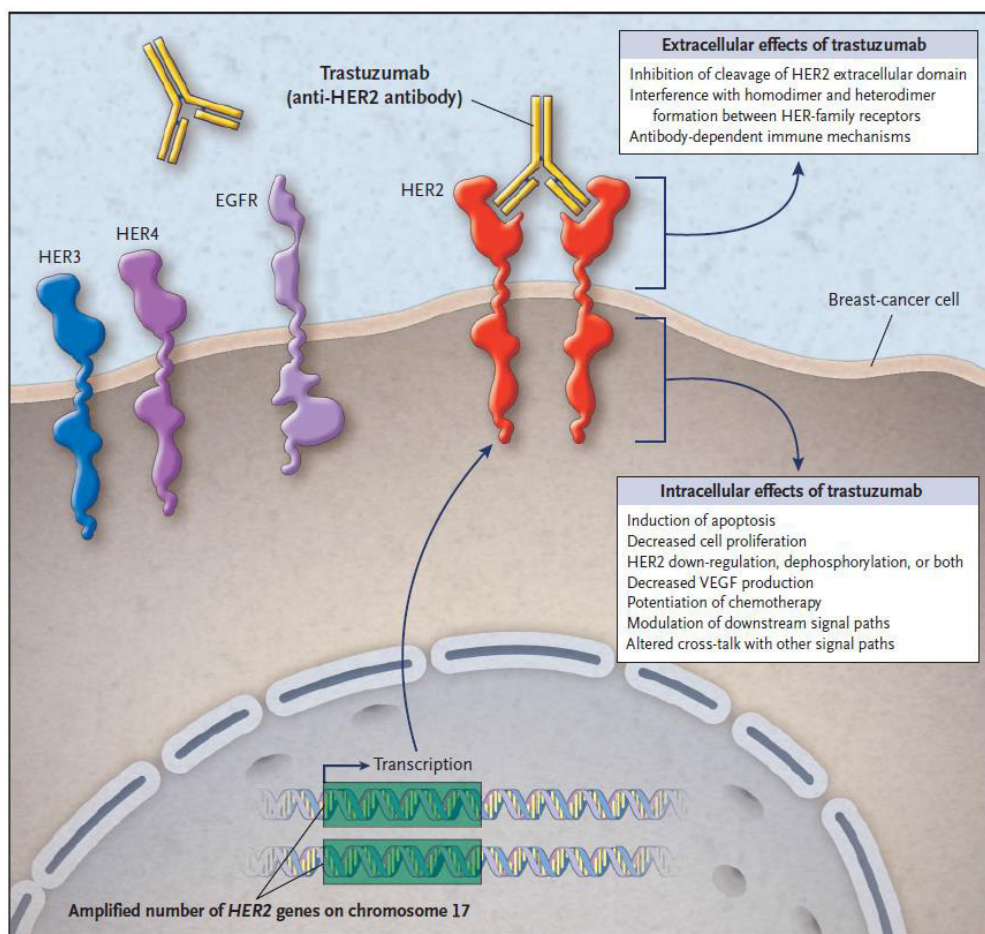


Figure 2 | Interactions between Trastuzumab and tumor cells. Reproduced with permission from Burstein, H. J., *N. Engl. J. Med.*, 2005, 353, 1652-1654, Copyright © 2005 Massachusetts Medical Society.

This work focuses on the synthesis and surface chemistry of nickel nanoparticles that can be conjugated to the trastuzumab antibody by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/ N-hydroxysulfosuccinimide (sulfo-NHS) linker chemistry and then be employed with the PlasMag measurement principle for protein biosensing. In chapter 2, first, the PlasMag technique for biosensing will be introduced and subsequently, the theoretical background for the template-based electrochemical synthesis of nickel nanorods will be presented. Then, different approaches for the stabilization of nanoparticles in colloidal dispersion and functionalization for the conjugation of antibodies will be discussed. A brief section on the theoretical basics of electron microscopy and the PlasMag measurement principle is included as well. In chapter 3, the experimental procedures and setups are presented, followed by the discussion of the obtained results in chapter 4. The thesis is concluded by an outlook on future applications and possible improvements for the described procedures.

2 Theoretical Section

2.1 Electrochemical Synthesis of Nickel Nanorods

The synthesis of nickel nanorods in colloidal suspension is a three-step process. First, nano-sized porous aluminium oxide (Al_2O_3) layers are produced using an anodizing process described by Masuda *et al.*^{59,60}. Then, the resulting pores are filled with nickel from a Nickel-Watts bath⁶¹ via pulsed electrodeposition⁶². In a final step, the nanorods are released from the aluminium oxide template and dispersed in water after several washing steps.

2.1.1 Formation of Porous Aluminium Oxide Layers

When pure aluminium is exposed to air, a surface layer of amorphous aluminium oxide of about 2-3 nm forms naturally, which prevents the aluminum from further oxidation in most environments⁶³. This process is called passivation and can be enhanced to create thicker aluminum oxide layers by passing a direct current through an electrolyte with the aluminium serving as the anode⁶⁴.

Fig. 3 provides a schematic illustration of the different processes during the anodization of the aluminium template. Due to the usage of conductive acids as electrolytes, the voltage drops almost completely across the aluminium oxide layer resulting in the formation of an electrical field, which allows ions to diffuse through the oxide layer without interrupting the oxidation process⁶⁴.

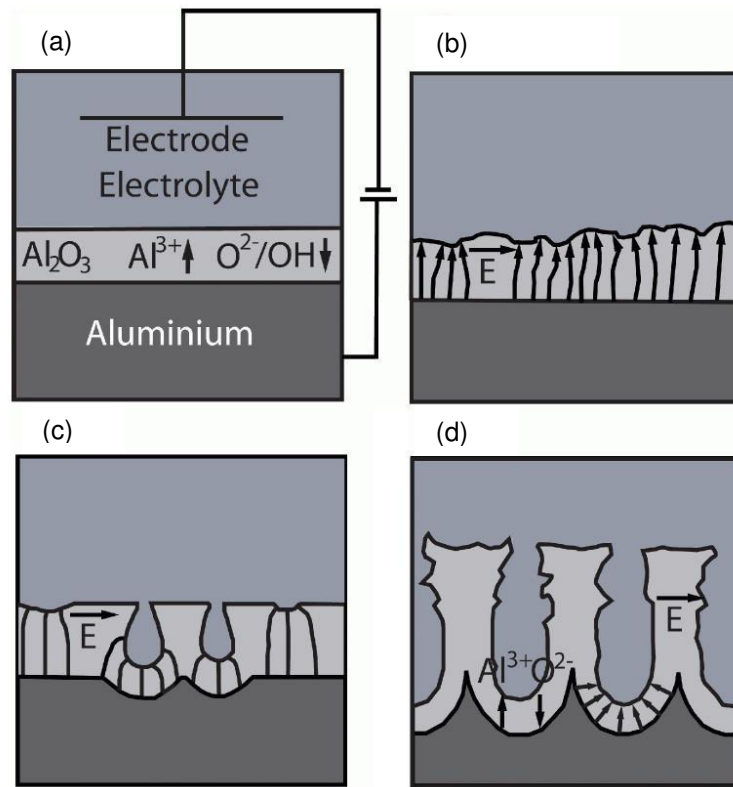
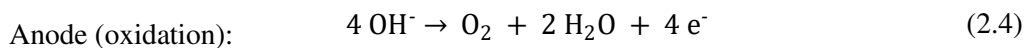
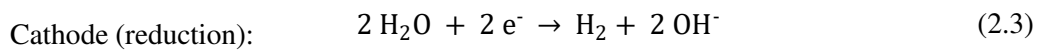


Figure 3 | Schematic illustration of the different processes during the anodization of the aluminium template. (a) Passivation of the aluminium template. (b) Formation of inhomogeneities on the aluminium oxide surface. (c) Development of pores in the aluminium oxide. (d) Pore growth. Reproduced and modified from Nielsch, K., “Hochgeordnete ferromagnetische Nanostabensembles – Elektrochemische Herstellung und magnetische Charakterisierung”, Martin-Luther-Universität Halle-Wittenberg, 2002.

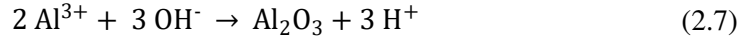
The electrolysis of water takes place as soon as an electric current is passed through water and therefore also happens at the electrolyte-Al₂O₃ interphase:



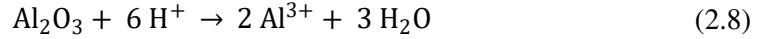
Due to the electrical field, the hydroxide (OH^-) and oxygen ions (O^{2-}) diffuse through the oxide to the Al_2O_3 -Al interface, where Al^{3+} ions are produced:



The OH^- and O^{2-} ions react with the Al^{3+} ions and form aluminium oxide:



Not all Al^{3+} ions are consumed for the formation of aluminium oxide and some of them transition to the electrolyte ⁶⁵. When strong acids are used as electrolytes, aluminium oxide is dissolved at the electrolyte- Al_2O_3 interphase concurrently:



The resulting H^+ ions then proceed to the cathode and form hydrogen gas (H_2):



As can be seen from the equations above and in Fig. 4, the relation between the formation of the aluminium oxide and dissolving of the oxide is crucial for the anodization process. When electrolytes like acetic acid or tartaric acid, which do not dissolve the oxide layer are employed, the electric field within the oxide layer decreases with increasing thickness of the layer, causing the ion diffusion to stop at a certain threshold. Thereby, a non-porous oxide layer with a limited thickness of approximately $1 \mu\text{m}$ is created ⁶⁶. By using electrolytes, that easily dissolve the oxide such as perchloric acid, the aluminium template gets electropolished ⁶⁷. However, to achieve the formation of a porous oxide layer, an electrolyte that mildly dissolves the oxide has to be chosen. Typical candidates for this procedure include sulfuric acid, phosphoric acid or oxalic acid ^{68,69}. When those acids are employed, the formation of aluminium oxide is favored over the dissolving of the oxide resulting in a porous oxide layer of up to $100 \mu\text{m}$ thickness.

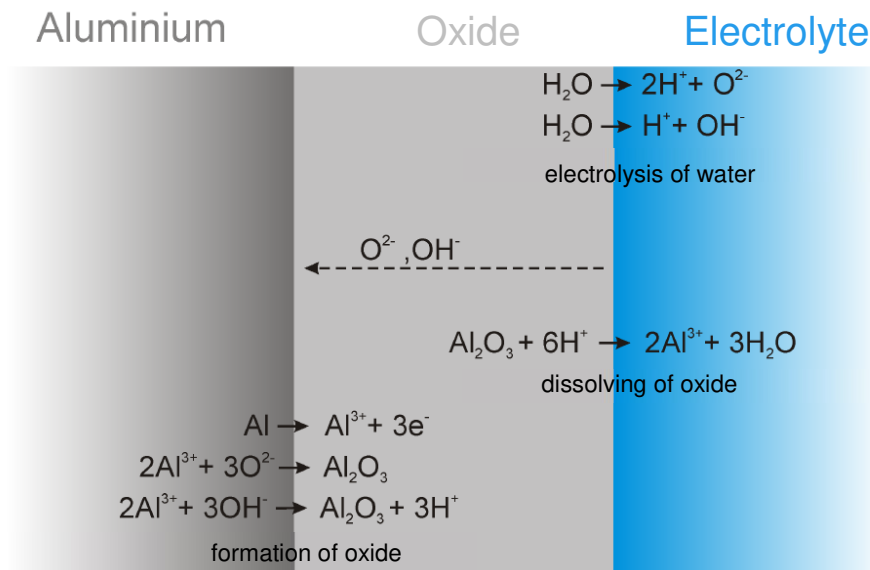


Figure 4 | Schematic illustration of the chemical reactions at the aluminium/aluminium oxide and aluminium oxide/electrolyte interface during the anodization process. Reproduced and modified from Krämer, F., "Präparation magnetischer Nanostäbe durch Templatverfahren und Untersuchung ihrer Mikrostruktur und magnetischen Eigenschaften", Universität des Saarlandes, Saarbrücken, 2011

When the voltage is applied at the beginning of the anodization process, only the naturally existing aluminium oxide layer of a few nanometers is present on the surface of the template, causing the current to reach a maximum as can be seen in Fig. 5, since the alumina layer only provides low resistance. Then, a compact oxide layer, the so-called "barrier oxide" is formed, which causes higher resistance and therefore results in a decrease in current. Due to the inhomogeneous and rough surface of the barrier oxide, the electric field lines focus in slots, which can be seen in Fig. 3, thereby increasing not only the electrolysis of water molecules but also the dissolving of oxide in slightly corrosive electrolytes like sulfuric acid ⁷⁰. While cavities on the surface of the barrier oxide are formed irregularly, the current reaches a minimum and stays mostly constant. The electric field lines stay focused at the bottom of the cavities and the dissolving of the oxide continues, resulting in a decrease in resistance and increase in current, while the pore formation starts. As soon as the ions are able to drift through the barrier oxide due to the dissolving process, formation of oxide also takes place and an equilibrium between these two processes is established. Since dissolving and formation of the oxide proceed simultaneously, a constant thickness of the barrier oxide and therefore no changes in the current are reached and pores are steadily formed.

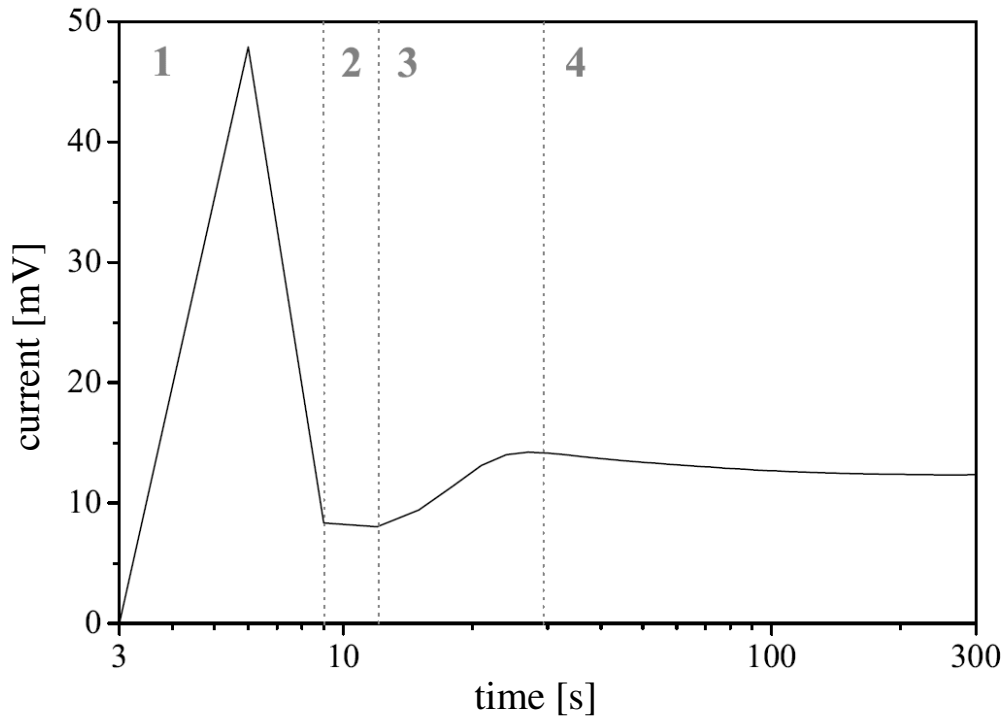


Figure 5 | Current as a function of time during the anodization process in sulfuric acid and a potential of 15 V. The formation of porous aluminium oxide can be categorized in 4 different phases, which are separated through vertical lines. Reproduced and modified from Günther, A., “Hydrodynamische Eigenschaften von magnetischen Nickelnanostäben in kolloidaler Suspension”, Universität des Saarlandes, Saarbrücken, 2011

Oxygen is incorporated for the pore formation during the anodization process, which leads to an increase in volume and further results in strain in the template. To minimize the mechanical stress, the pores adapt a hexagonal order, which is depicted in Fig. 6.

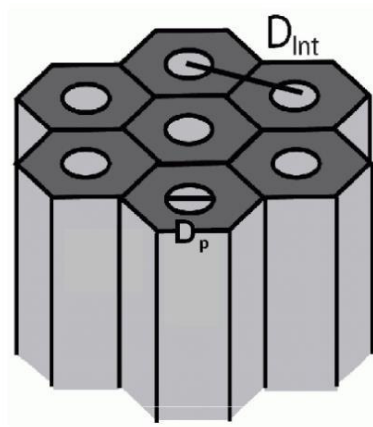


Figure 6 | Structure of cylindrical aluminium oxide pores adapting a hexagonal order. The pore diameter is denoted as D_P and the center-to-center distance is denoted as D_{Int} . Reproduced from Krämer, F., “Präparation magnetischer Nanostäbe durch Templatverfahren und Untersuchung ihrer Mikrostruktur und magnetischen Eigenschaften”, Universität des Saarlandes, Saarbrücken, 2011

The center-to-center distance D_{Int} between two pores of the hexagonal structure can be estimated with the empirical correlation proposed by Nielsch⁷¹, with U_{Ox} being the applied anodization voltage:

$$D_{Int} = 2.5 \frac{nm}{V} \cdot U_{Ox} \quad (3.0)$$

While the order of the hexagonal structure at the growth front increases in the ongoing anodization process, the pore channels at the surface are inhomogeneous and unordered⁷². Since this would result in nanorods differing substantially from the ideal cylindrical form, a second anodization process according to Masuda *et al.* is conducted as will be discussed below^{59,60}.

To begin with, the first anodization of the aluminium template is performed as described above and results in a highly ordered, hexagonal pore structure at the growth front. The aluminium oxide is then chemically selective dissolved and leaves an imprint of the pore structure at the template. Fig. 3 (d) shows the pore channels prior to the described dissolving process. Then, the second anodization is performed under the same conditions (temperature, electrolyte) as the first one, resulting in homogeneous, highly ordered, hexagonal pores instead of inhomogeneous and unordered pore channels in the aluminium oxide due to the preexisting pore imprint, which replaces the inhomogeneities of the first anodization.

The insulating barrier oxide is also crucial for the electrodeposition of nickel, since it separates the conducting aluminium foil from the pores. To accomplish the electrodeposition of nickel into the pores, a sufficiently high voltage must be applied to enable electrons to tunnel through the aluminium oxide barrier. Therefore, the barrier has to be as thin as possible without endangering the mechanical stability of the pore structure. To achieve a high filling level of the pores, the thickness of the barrier oxide has to be reduced during the anodization. Nielsch *et al.* developed a current-limited thinning procedure, where the current is gradually reduced at the end of the second anodization⁶². After each reduction of the current, a new equilibrium between oxide dissolving and formation is established. A change in the applied voltage leads to a change in the thickness of the barrier oxide d_{Ox} , which can be described by the empirical formula, with σ being a proportionality factor dependent on the electrolyte.

$$d_{Ox} = \sigma \cdot U_{Ox} \quad (3.1)$$

According to equation (3.0), the distance of the pores is also proportional to the anodization voltage, resulting in a growth with changed pore surface density due to the ion passing during the barrier thinning process. This can lead to a subdivision of pores at the growth front and the formation of secondary and tertiary pores, if the voltage is further reduced. These so-called dendrites cause deviations from the ideal cylindrical rod shape and are

therefore unwanted just as rough pore channels, which can be smoothed by widening the pores, which will be discussed in the following paragraphs.

The pores consist of two different layers of aluminium oxide ⁷³. One layer is made from pure aluminum oxide and adapts a hexagonal honeycomb structure, while electrolyte-contaminated aluminium oxide can be found between the hexagonal structure and the cylindrical pore in the center. The occurrence of this double-layer-structure is dependent on the electrolyte.

The inner layer of the pore channels consists of the contaminated aluminium oxide and shows severe inhomogeneities especially at the electrolyte-interface, which can cause irregular rod shapes. By inserting the template into a bath filled with an appropriate acid, the impure oxide can be partially dissolved without damaging the pore structure. This process not only homogenizes the inner layer of the pore channels but also widens the diameter of the pores. The longer the template stays in the acid bath, the more uniform and the greater the diameter of the pores get.

2.1.2 Pulsed Electrodeposition of Nickel into Porous Aluminium Oxide Layers

For the electrodeposition, an electrochemical cell is filled with an electrolyte, containing the desired metal ions, in this case nickel cations (Ni^{2+}), similar to the setup for the anodization process. Due to the insulating barrier oxide it is difficult to deposit metal ions into the pores, since the application of high voltages might cause the destruction of the oxide layer. Nielsch further developed Caboni's principle of alternating current (AC) deposition for current-pulsed deposition of metals into pores of the aluminium oxide ⁷¹. To achieve electrodeposition, a voltage has to be applied, which is high enough to enable tunneling of electrons. This voltage is proportional to the thickness of the oxide layer. Due to the homogenization of pores after the second anodization, the barrier oxide is thin enough to apply a voltage which allows tunneling of electrons without damaging the oxide layer. Instead of applying a constant voltage, several sequences of different current pulses were employed, consisting of a short, constantly negative pulse, followed by an even shorter, constantly positive pulse and a longer break at the end of the sequence, as demonstrated in Fig. 7.

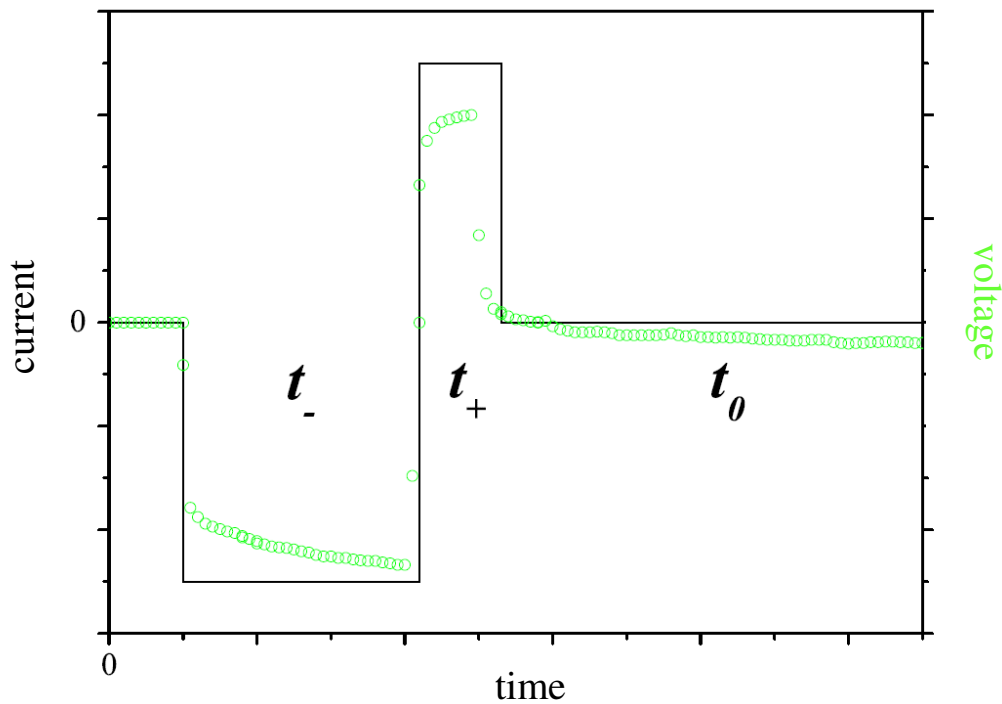


Figure 7 | Current pulses (black) and the respective voltage characteristic (green) during the electrodeposition of nickel into the aluminium oxide pores. One pulse sequence consists of a negative current pulse for the period t_- , followed by a positive current pulse for the period t_+ and a longer resting period t_0 , where no current is applied. Reproduced and modified from Günther, A., “Hydrodynamische Eigenschaften von magnetischen Nickelnanostäben in kolloidaler Suspension”, Universität des Saarlandes, Saarbrücken, 2011

The metal is deposited into the pores during the first, negative, current pulse as a result of the positive charge of the metal cation and the cathodic nature of the aluminum oxide membrane. Due to the formation of a concentration gradient and the charging of the barrier oxide, similar to a capacitor, the potential slightly increases during the pulse. During the second, positive pulse, the discharging of the aluminium oxide takes place as well as the dissolving of homogeneities in the barrier oxide, which is why this pulse has to be kept short to avoid dissolving of the deposited nickel nanorods. During the subsequent pause, the concentration gradient and depletion of ions at the bottom of the pores is compensated by the diffusion of metal ions from the solution into the pores, which is a thermally driven process and therefore time-consuming. If the pause interval is too short, the concentration gradient is still existent and too little metal ions are present in the pores causing the deposition process to stop. Instead, the formation of hydrogen gas becomes the favored reaction, which results in a change of the pH value and subsequently causes undesired side reactions. Homogeneous filling of the pores thereby requires a constant pH value, which can be achieved by using buffered nickel plating solutions, *e.g.* Nickel Watt’s bath^{61,74}. By choosing a pause interval, which is long enough to avoid the described obstacles, the same conditions can be created prior to each deposition pulse. The length of the nanorods can then be influenced by the number of pulse sequences.

2.1.3 Removal of Nickel Nanorods from the Aluminium Oxide Template

In a last step, the magnetic nanoparticles are released from the aluminium oxide template and stabilized in colloidal dispersion. Therefore, the oxide must be chemically selective dissolved to avoid damage of the nanorods. In most cases, alkaline solvents exhibit the desired behavior. Furthermore, agglomeration through attractive Van-der-Waals and magnetic dipole-dipole interactions must be prevented, because it would lead to agglomeration and sedimentation of the nanoparticles, ultimately. To ensure the stability of the colloidal dispersion, surfactants (**surface active agent** $\triangleq$ surfactant) like poly(*N*-vinylpyrrolidone) (PVP) are added. Surfactants are amphiphilic molecules containing both hydrophobic and hydrophilic groups, which adsorb to the surface of the particle and prevent agglomeration due to electrostatic, steric or electrosteric repulsion.

2.2 Stabilization of Colloidal Nanorod Dispersions

A colloid is typically defined as a two-phase system consisting of a continuous phase (dispersion medium) and the dispersed phase (particles or emulsion droplets). The size of the dispersed particles commonly ranges from 1 nm to 1 μm . Suspensions (solid/liquid), emulsions (liquid/liquid) and foams (gas/liquid) are the most prominent examples for colloidal dispersions, although there are many other types. Because of their dimensions, magnetic nanoparticles can be dispersed in a continuous phase and a stable colloidal dispersion can be obtained, which combines the properties of usual colloidal dispersions and the magnetic properties of the dispersed particles.

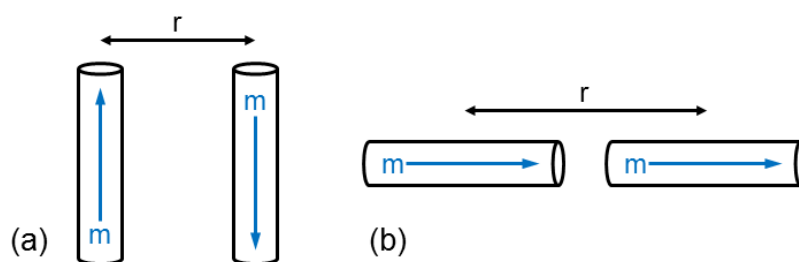


Figure 8 | Possible nanorod configurations for cylindrical particles with fixed magnetization directions ($\mathbf{m}$). The nanorods act as magnetic dipoles at a distance r . (a) Favored parallel nanorod alignment with antiparallel magnetization directions due to a minimal dipole-dipole-energy. (b) Tip-to-end nanorod alignment with magnetizations oriented in the same direction. Reproduced with permission from Schrittwieser, S., “Modeling and Development of a Biosensor Based on Optical Detection of the Rotational Dynamics of Hybrid Nanoparticles”, Bielefeld University, AIT Austrian Institute of Technology, 2014.

Colloids are stabilized to protect them from aggregation and/or phase separations. In general, colloidal dispersions are considered stable, if the thermal energy is greater than the

sum of all positive (particle attracting) and negative (particle repelling) interactions^{75,76}. For the application of the magnetic nanoparticles in the PlasMag system, the nanorods must be stable in aqueous buffer solution. It is crucial to overcome attracting magnetic interactions originating mainly from dipole-dipole interactions. For cylindrical nanorods, the geometric anisotropy results in the alignment of the magnetic moment parallel to the main cylinder axis and two possible geometric nanorod orientations exist, which are depicted in Fig. 8. In one configuration, the magnetization directions of the nanorods are antiparallel to each other, while the magnetization of the nanoparticles adapting a tip-to-end alignment is pointing in the same direction in the second configuration⁷⁵. The dipole-dipole energy of the parallel configuration is smaller than the one for the tip-to-end configuration and thus, favored during agglomeration⁷⁵. For nanorods with the same absolute value of the magnetic moment and antiparallel configuration, the magnetic dipole-dipole energy is described by the following equation.⁷⁵, with μ_0 being the magnetic permeability and r being the distance between two nanorods:

$$U_{dd} = - \frac{m^2 \cdot \mu_0}{4 \cdot \pi \cdot r^3} \quad (3.2)$$

The stabilization with electrostatic forces is a method to overcome the attracting magnetic dipole-dipole interactions for particle dispersions in solution, due to the mutual repulsion of like electric charges. The surface charge is caused by attached charged groups or a naturally developed charged surface in a distinct medium⁷⁷. Since different phases have different charge affinities, an electrical double layer, which consists of counterions balancing the charge of the particle in solution near the surface is formed at any surface. The first layer, which is also called Stern layer is composed of counterions which have the opposite charge of the particle and is tightly attached to the particle surface. The potential across the Stern layer is called Stern potential and linearly decreases with distance from the particle surface. After the first, strongly bound layer a second, diffuse layer of fixed and weakly bound ions follows. The associated potential U_{DL} drops exponentially with distance d and the characteristic Debye-Hückel parameter κ :

$$U_{DL}(d) = e^{-\kappa d} \quad (3.3)$$

The Debye-Hückel length or Debye length is accessible by the inverse of κ , which is given by

$$\kappa^{-1} = \left(\frac{e_0^2}{\epsilon_r \cdot \epsilon_0 \cdot k_B \cdot T} \cdot \sum_i c_i \cdot z_i^2 \right)^{-\frac{1}{2}} \quad (3.4)$$

where e_0 is the elementary charge, ϵ_r the relative permittivity of the medium, ϵ_0 the vacuum permittivity, k_B the Boltzmann constant, T the temperature, z_i the charge number and c_i the concentration of the charged species. The ionic strength IS can be correlated to the sum in equation 3.5:

$$IS = \frac{1}{2} \cdot \sum_i c_i \cdot z_i^2 \quad (3.5)$$

The Zeta potential (ζ -potential) describes the electrokinetic potential in colloidal dispersions. As can be seen in Fig. 9, it is defined as the potential decay between the solid surface and the bulk liquid phase at the shear plane ⁷⁸.

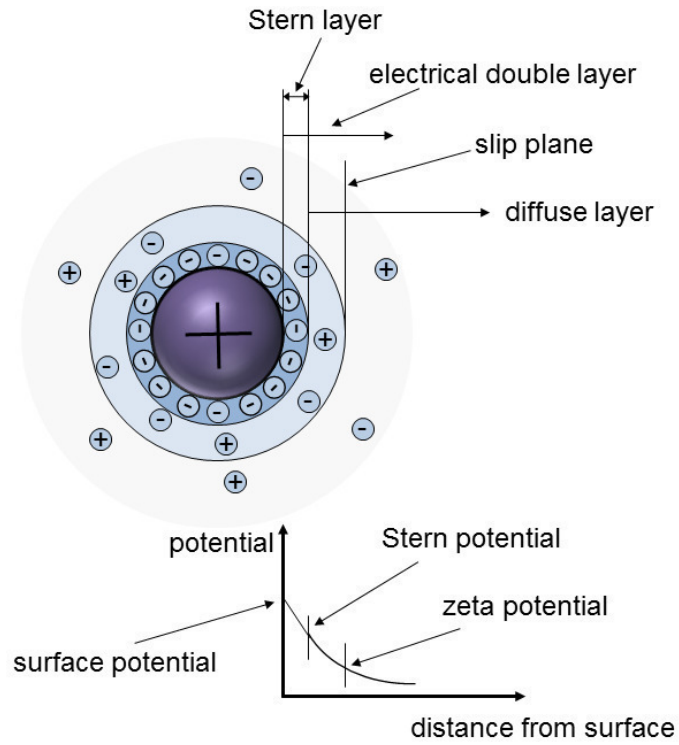


Figure 9 | Schematic illustration of the electrical double layer and potentials of a nanoparticle with positive surface charge. The Stern layer is the first layer on the particle surface with strongly fixed counter ions. The following diffuse layer is composed of fixed and weakly bound ions with positive and negative charge. Bound immobile ions are separated from weakly bound ones through the slip plane, which determines the zeta potential. At first, the potential decreases linearly from the surface to the Stern potential, from where it drops exponentially with increasing distance from the particle surface. Reproduced with permission from Schrittwieser, S., "Modeling and Development of a Biosensor Based on Optical Detection of the Rotational Dynamics of Hybrid Nanoparticles", Bielefeld University, AIT Austrian Institute of Technology, 2014.

The higher the zeta-potential, the stronger is the electrostatic repulsion between particles and the more stable the dispersion system becomes. The range of the electrostatic interaction between two particles in electrolytes is determined by the thickness of the electrical double layer, and consequently the Debye length. The strength of the electrostatic interaction between charged particles is defined by the surface charges, which are weakened by the electrical double layers. If the ionic strength is given, the charge of the particle surface determines the electrostatic interaction ⁷⁹.

Van der Waals interactions take place on a short range due to a strong distance dependence of r^{-6} and exist due to the polarization of electron clouds of two interacting nuclei ^{80,81}. There are three different forms of van der Waals interactions. Firstly, the Keesom interaction, which is a force between two permanent dipoles. Secondly, the Debye force, which is the interaction of a permanent dipole with an induced dipole and thirdly, the London force between two polarizable molecules that is based on spontaneous oscillations of electron clouds, which result in temporary dipoles. Usually, the van der Waals interaction is an attractive force proportional to the surface area of contact, but because of its short range, it can be attenuated by steric hindrance as discussed in the following paragraph ^{80,81}.

Instead of using electrical charge, repulsion between dispersed particles can also be developed by means of absorbed polymer coatings on the particle surface. This approach is based on steric stabilization and defined as the binding (adsorption or grafting) of non-ionic molecules to the particle surface. It can be achieved by attaching large molecules (*e.g.* polymers) to the surface of nanoparticles, thus preventing the particles from getting close ^{80,82}. In contrast to electrostatic stabilization, which is mostly restricted to dilute particle dispersions and sensitive to electrolytes, steric stabilization is also applicable in high particle concentrations and not sensitive to electrolyte solutions ⁸⁰. While electrostatic stabilization is based on kinetics, steric stabilization relies on thermodynamics and therefore allows easy redispersion of already agglomerated particles ^{80,82,83}.

The electrosteric stabilization approach combines the effects of the electrostatic and steric stabilization methods and has been described, for example, by Lourenco *et al.* ⁸⁴.

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory describes the stability of charged colloidal particles based on the combination of electrostatic repulsion and van der Waals interactions, but it has to be extended to include magnetic dipole attractions, non-DLVO forces and steric repulsion terms derived from the presence of surfactant or polymeric layers. Thus, the stability of colloidal dispersions depends on the nature of the continuous phase, the dimension of the particles, surface characteristics and the presence of stabilizing agents ^{85,86}.

2.3 Surface Functionalization of Nickel Nanorods

Prior to surface modifications, it is a prerequisite to separate the nanorods from the nanoporous template in which they were synthesized. Aluminium oxide and polycarbonate are commonly used template materials for electrodeposition, which can be removed without damaging the nickel nanorods through chemically selective etching. The aluminium oxide template can be dissolved in sodium hydroxide solutions or mixtures of chromic acid and phosphoric acid, while dichloromethane is usually employed for the dissolving of the polycarbonate membrane⁸⁷.

Surface modification of nickel nanorods is not only possible after the removal of the template, but also before electrodeposition by modifying the walls of pore channels to yield surface functionalized nanorods^{88–90}.

In the case of inorganic particles, functional groups on the surface can be present intrinsically (*e.g.* OH groups on the surface of metal oxide particles) or due to ligands (surfactants) introduced during the synthesis. Surface functionalization of nanoparticles is often required to facilitate their re-dispersion in various solvents, biological recognition or cellular uptake. By attaching hydrophilic or hydrophobic molecules, including small molecules, surfactants, synthetic oligomers, polymers and bio-macromolecules, nanoparticles can be functionalized specifically for their desired application⁸⁸. Through the coupling of nanoparticles to functional molecules or biomolecules like nucleic acids, proteins, cells, enzymes, etc., the required additional functionalities for cell labeling⁹¹, sensing¹², diagnostic⁹² or molecular imaging⁹³ applications can be introduced. The process of chemically joining two or more molecules by a covalent bond, with at least one of the molecules being a biomolecule like nucleic acids, proteins, cells, enzymes etc. is known as bioconjugation⁹⁴. Methods for mild and site-specific derivatization of biomolecules have been developed for applications such as ligand discovery, disease diagnosis and high-throughput screening owing to the existence of chemoselective reactions enabling bioconjugation under physiological conditions^{94–96}. The functionalization methods can be roughly divided into physical and chemical approaches^{97,98}.

The chemistry behind the functionalization approaches is primarily based on noncovalent binding and covalent conjugation. Conjugation through noncovalent interactions is achieved by the attachment of specific secondary ligands and mainly performed by affinity-based receptor-ligand systems via electrostatic interactions, π - π stacking and entrapping biomolecules in biocompatible films like phospholipids, polymer, DNA, etc⁹⁹. The direct binding of molecules to reactive ligands on the nanoparticle surface by covalent conjugation includes chemisorption through thiol derivatives, bifunctional linkers/mediators, adapter molecules such as streptavidin and biotin and coordinative binding.

Prior to covalent coupling, the particle surface can be chemically modified to offer reactive functional groups. Exemplary, the process of covering a surface with organofunctional alkoxy silane molecules is called silanization and results in a thin silica layer on the particle, which can provide protection against the environment. Silane head groups form covalent bonds with hydroxyl groups present on the surface of the metal oxide substrate in a self-

assembly process ^{100,101}. Through the use of aminoalkylsilanes and mercaptoalkylsilanes as coupling agents, surface groups can be introduced for further covalent reactions.

Another approach is the functionalization with PVP, because its pyrrolidone group provides a high polarity, which results in a very good solubility in water and other polar solvents ¹⁰². The long-chained polymer possesses a high affinity to nickel oxide promoting its adsorption through the hydrophilic head group to the surface of the nickel nanorods during the dissolving of the oxide in the nanorod fabrication process. Through the PVP coating, steric repulsions between the particles are provoked, which ultimately prevent agglomeration.

Furthermore, nickel nanorod surface modification methods based on carboxylic acids are widely employed and based on the high affinity to the native nickel oxide layer which is present on the bare nickel nanorods ^{103–106}. During the binding process of a carboxylic acid to a metal surface, metal-carboxylate salts may be generated or the carboxyl group might adhere to the metal with a concurrently occurring proton transfer from the carboxyl group to an oxygen atom on the metal surface, which is provided by the native oxide layer in the case of nickel nanorods ¹⁰³.

Some of the most frequently used reactions for covalent functionalization, as can be seen in Fig. 10, include ⁹⁷:

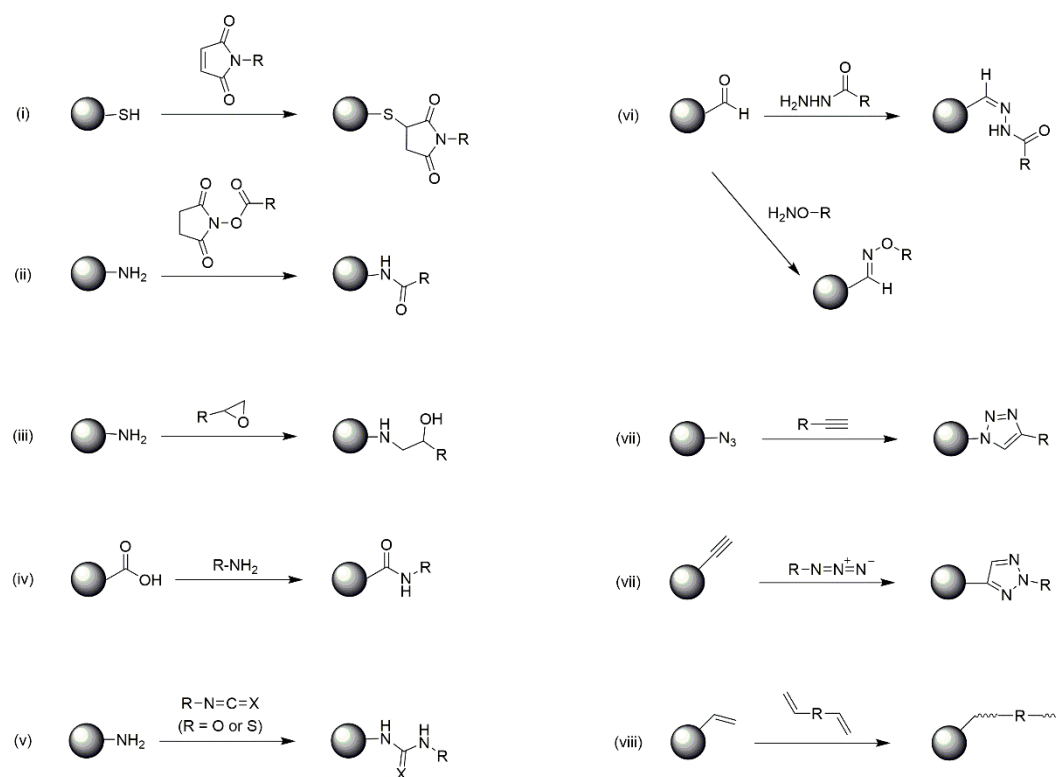


Figure 10 | Strategies for covalent functionalization for nanoparticles.

- (i) the Michael addition of thiol groups to maleimides
- (ii) the amidation of surface amino groups with NHS esters derivatives
- (iii) the reaction between amino groups and epoxides
- (iv) the amine bond formation between amino and carboxylic groups by employing coupling reagents such as EDC, NHS, sulfo-NHS
- (v) the reaction of amines with isocyanate derivatives
- (vi) the imine formation
- (vii) Click chemistry reactions between azides and alkynes
- (viii) Metathesis reactions including ring-closing metathesis, cross metathesis and ring opening metathesis polymerization (ROMP)

Depending on the available functional groups on the nanoparticle surface, different approaches for further modifications, as demonstrated above, can be chosen. Exemplary, thiol- and amine groups can be introduced by coating the particles with mercaptoalkylsilanes and aminoalkylsilanes. Furthermore, polymers offer a broad range of reactive functional groups, which can be employed for covalent functionalization.

2.4 Scanning Electron Microscopy

The unaided eye provides a resolution of about 0.1 mm, while optical microscopy has the limit of resolution of approximately 200 nm by enlarging the visual angle through optical lenses ^{107,108}. With a scanning electron microscope (SEM), resolutions of 1-20 nm depending on the instrument can be achieved. The resolution is limited by the wavelength of the light (electrons) that is employed for experimental observation. This is the so-called Abbe limit, which results in a resolution that is roughly the half of the employed wavelength ^{107,108}. De Broglie postulated that every particle or matter may be partly described in terms not only of particles, but also of waves and therefore has a dual character: It can be either regarded as a moving particle or as radiation with an associated wavelength ^{109,110}. The resolution of an electron microscope is limited by the probing wavelength and the numerical aperture. Since the wavelength of electrons is much smaller than that of photons, a resolution of approximately 0.1 nm can be achieved, but it is still limited due to the objective lens system.

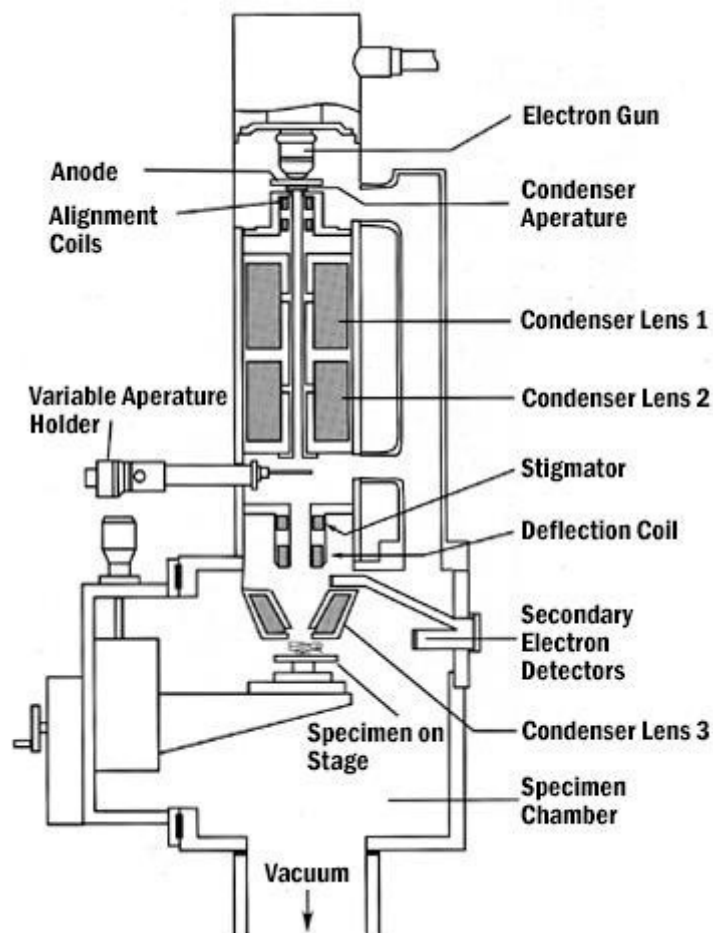


Figure 11 | Schematic setup of a Scanning Electron Microscope. Reproduced from ¹¹¹.

The scanning electron microscope (SEM) belongs to the group of electron microscopes, which operate like conventional light microscopes, but the light source is replaced by an electron beam. It is used for the examination and analysis of the microstructure morphology

and chemical composition characterizations by irradiating a both electrically conductive and electrically grounded sample with a fine electron beam, which causes the emission of secondary electrons from the sample surface due to interactions between the electrons and atoms at various depths within the sample. The surface topography and composition are accessible through two-dimensional scanning of the electron beam over the surface in a raster scan pattern where the beam's position is combined with the detected signal to acquire images of the detected secondary electrons in most cases ¹⁰⁸.

The SEM typically consists an electron optical system to produce an electron beam, a sample stage to ensure correct placement of the specimen, mostly a secondary-electron detector to register secondary electrons, which are all kept at high vacuum (10^{-3} - 10^{-4} Pa), as well as an image display unit and an operation system as can be seen in Fig. 11 ¹⁰⁸.

Electron guns are required to produce a stable electron beam with high current, small spot size, adjustable energy and small energy dispersion. Several types of electron guns can be used in SEM systems depending on the application. In the case of a thermionic emission gun, thermoelectrons are emitted from a filament (cathode) made from tungsten wire by heating the filament at high temperature (approximately 2,800 K). Then, the thermoelectrons are focused as an electron beam by passing through a hole the metal plate (anode) through the application a positive voltage (1-30 kV) to the anode. The so-called Wehnelt electrode is placed between the cathode and the anode and enables focusing and adjustments of the current of the electron beam through the application of a negative voltage. The finest point of the focused beam is called crossover and regarded as the actual electron source with a diameter of 15-20 μm . Other electron guns include single crystal lanthanum hexaboride (LaB_6) cathodes, field emission guns or the Schottky-emission electron guns ¹⁰⁸.

The beam is then further focused to a spot of approximately 0.4-5 nm in diameter by condenser lenses and passes through pairs of scanning coils or deflector plates in the electron column, to be deflected by the final lens so that raster scans of the sample surface can be performed. The primary electron beam interacts with the sample which leads to a decrease of energy of the electrons by repeated random scattering and absorption within a teardrop-shaped volume of the sample, the so-called interaction volume, which ranges from less than 100 nm to about 5 μm into the surface. This energy exchange leads to elastic and inelastic scattering and the emission of electromagnetic radiation of the electrons. Elastic scattering is caused by the deflection of the incident electron by the atomic nucleus or by outer shell electrons of sample atoms. A negligible energy loss during the collision and a wide-angle directional change of the scattered electron are typical for this kind of scattering. Electrons that are elastically scattered through an angle of more than 90° are called backscattered electrons (BSE) and can be exploited for signal detection. The backscattered electrons are sensitive to the composition of the sample, because the larger the atomic number of the constituent atoms in the sample, the larger is the BSE yield due to more positive charges on the nucleus. For this reason, an area consisting of heavier atoms appears bright in the BSE image which can thus be suitable for observing compositional differences. A variety of interactions between the incident electron and electrons or atoms of the sample result in inelastic scattering in which a substantial amount of energy from the electron beam is transferred to the sample atom. This energy transfer leads to the excitation of sample electrons during the ionization of the sample atoms and ultimately creates secondary

electrons (SE), which possess energies less than 50 eV and can also be utilized in signal detection. Aside from the described signals used in sample imaging, several other signals are produced when an electrons beam strikes a sample including the emission of characteristic x-rays, Auger electrons and cathodoluminescence ¹⁰⁸.

In the most cases, low-energy secondary electrons ejected from the k-shell of the sample atoms are detected upon collision with the electron beam. They originate within a few nanometers from the sample surface due to their low energy and mark the position of the electron beam as well as giving topographic information with good resolution ^{108,112}. An Everhart-Thronley detector is used for the detection of these secondary electrons. This detector consists of a scintillator inside a Faraday cage inside the sample chamber of the microscope ¹¹³. The secondary electrons are collected by attracting them towards a positively charged grid and then accelerated towards the scintillator. The positive voltage of the scintillator is high enough to accelerate the incoming electrons to where they are sufficiently energetic to hit the scintillator and thereby generate photons, which then travel through a light guide to a photomultiplier tube outside the SEM for amplification, processing and image display ¹⁰⁸.

2.5 PlasMag Measurement Principle

The measurement method relies on optical observation of the dynamics of antibody-functionalized magnetic nanoparticles ('nanoprobes' or 'nanorods') excited in solution by a rotating magnetic field (RMF) and is illustrated in Fig. 12. The elongated nanoparticle shape causes anisotropic polarizability which leads to optical absorption and scattering factors that strongly depend on the orientation of the nanorods relative to the polarization direction of the incident light, thus, allowing the optical detection of the nanoprobe alignment in the sample solution. The optical response of the nanoprobes can be amplified by a noble metal shell covering the magnetic nanorod, which enables the excitation of localized surface plasmon resonances. During the rotational motion of the nanorod, its hydrodynamic volume results in a drag torque, which causes the orientation of the nanorod to lag behind the applied RMF by a characteristic phase lag angle. When the target protein binds to the antibody-functionalized nanoparticle, its hydrodynamic radius increases, thereby creating a signal which is distinguishable from the signal caused by "naked" nanoparticles. The momentary vector of the applied RMF is correlated to the measured alignment of the nanoprobes, thus permitting the deduction of their mean phase lag angle relative to the applied field direction.

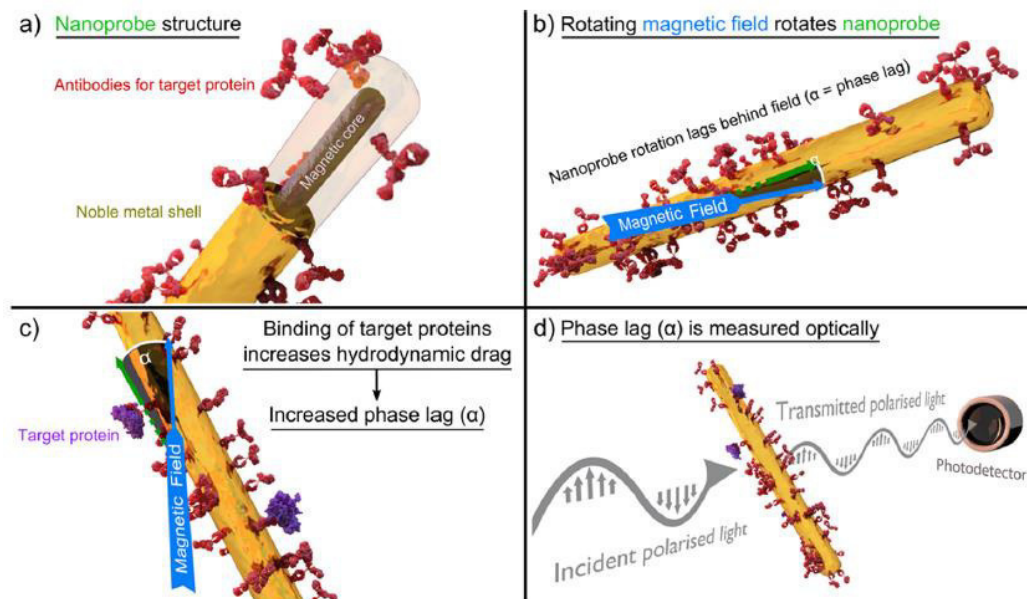


Figure 12 | Concept of the PlasMag measurement principle illustrated with noble metal coated nanorods. (a) Magnetic core nanorod functionalized with an antibody (nanoprobe). (b) A rotating magnetic field is used to agitate nanoprobe in the sample fluid. The hydrodynamic drag in the sample fluid causes the nanoprobe to follow the magnetic field at a phase lag angle α . (c) The binding of target molecules to the nanoprobe results in an increase in the thickness of the hydrodynamic shell and therefore also leads to an increase in α . (d) By measuring the transmission of polarized light, the phase lag angle α can be accessed. The intensity of the signal depends on the nanoprobe orientation with respect to the polarization direction. Reprinted with permission from Schrittwieser, S. et al., *ACS Appl. Mater. Interfaces*, 2016, 8 (14), 8893–8899, Copyright © 2016 American Chemical Society

As can be seen in Fig. 13, the PlasMag setup used for measurements of the rotational behavior of magnetic nanoparticles in external magnetic fields consists of three main parts, which are the rotating magnetic field generating component, the optical detection unit for measurements of the transmission of nanoparticle dispersions and an interface for electronic data acquisition.

The rotating magnetic field is provided by two perpendicularly aligned Helmholtz coils which are fed with two sinusoidal currents possessing a relative 90° phase shift to each other. Here, a circular homogeneous rotating magnetic field is obtained as long as each coil's current is in phase with its corresponding magnetic field. Shunt resistors are used for the monitoring of the currents, which enable the calibration of the amplitudes and the phases of the two currents, hence, the adjustment of the rotating magnetic field until the desired strength at the frequency of interest is obtained. The current through each coil is measured through the voltage drop across the precision shunt resistors. The two sinusoidal currents are generated by the soundcard of the measurement computer and then further amplified by a commercially available two channel audio amplifier that is able to produce high currents at low frequencies.

A fiber coupled laser diode produces a collimated, linearly polarized light beam with a wavelength of 785 nm, which passes through the sample solution in a cuvette in the center of the setup in the optical part of the setup. This incoming light possesses a polarization extinction ratio of 25 cB and is polarized parallel to the magnetic field that originates from

the small coil pair. The transmitted light is then collected by a focusing lens attached to a second fiber that passes the transmitted light to a silicon photodetector.

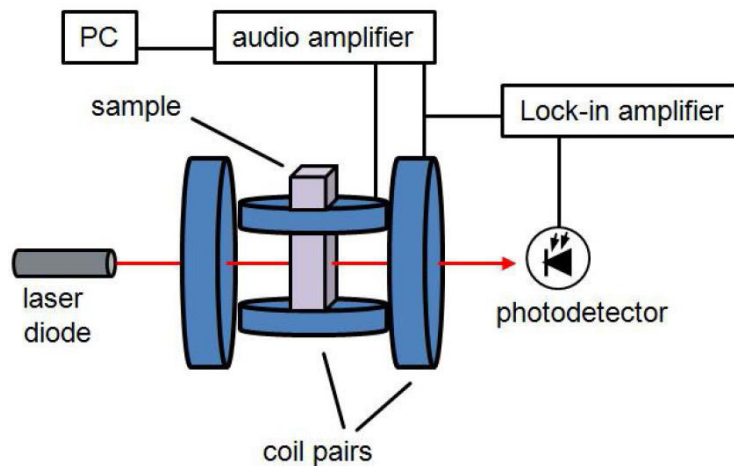


Figure 13 | The PlasMag setup consists of two perpendicular Helmholtz coils with the sample cuvette in their center. To generate a rotating magnetic field, the coils are fed by currents generated by the sound card of a computer and an audio amplifier. The transmission of polarized light generated by a laser diode through the sample is measured with a photodetector. The phase lag can then be calculated through the comparison of the optical with the magnetic signal by the lock-in amplifier. Reproduced with permission from Schrittwieser, S., "Modeling and Development of a Biosensor Based on Optical Detection of the Rotational Dynamics of Hybrid Nanoparticles", Bielefeld University, AIT Austrian Institute of Technology, 2014.

A lock-in amplifier records the generated signal and determines the phase lag between the magnetic field and the long particle axis. The output channel of the photo detector is connected to the signal input of the lock-in amplifier and the reference input is fed by the signal generated at the shunt resistor of the Helmholtz coil with its axis aligned parallel to the polarization direction of the incoming laser light beam. Due to the elongated symmetry of the nanorods, the second harmonic of the reference signal is measured by the lock-in amplifier. Measurements of the signal amplitude and phase are carried out as a function of the frequency and the magnitude of the applied rotating magnetic field. The signal output for the generation of the currents fed to the coils is controlled by a custom LabView program, which is responsible for the data acquisition as well.

The main components of the setup are mounted on an optical air damped breadboard and the whole setup is enclosed in a small lightproof box to meet laser safety regulations. Additionally, the optical part of the setup is mechanically decoupled from the Helmholtz coils to minimize mechanical vibrations.

3 Experimental Section

3.1 Materials and Methods

3.1.1 Electrochemical Synthesis of Nickel Nanorods

The following chemicals and devices were purchased from commercial sources and employed for nanorod synthesis: Power Supply Bipolar High BOP50-20MG (Kepco, Inc., USA), Agilent 34401A, 6 ½ Digit Digital Multimeter (Agilent Technologies, Inc., USA), F12 Cooling Unit (Julabo GmbH, Deutschland), MCS66 Programmable Hotplate Magnetic Stirrer (CAT Scientific, USA), MCS67 Programmable Hotplate Magnetic Stirrer (CAT Scientific, USA), MCS78 Programmable Hotplate Magnetic Stirrer (CAT Scientific, USA), Autolab PGSTAT30 potentiostat (Metrohm AG, Switzerland), Ultrasonic cleaner (45 kHz, 100 W; VWR, Austria), stainless steel foil (AISI 304L, 50×50×0.5 mm; GoodFellow, USA), platinum wire (0.5mm diameter, annealed, 99.95% (metals basis), 25 cm; Alfa Aesar, USA), nickel wire (0.5mm diameter, Puratronic®, 99.995% (metals basis), 1 m; Alfa Aesar, USA), copper wire (0.5mm diameter, Puratronic®, 99.999% (metals basis), 5 m; Alfa Aesar, USA), Ag/AgCl reference electrode with flexible wire connector (MW-2030, RE-6; BASi Inc., USA), Float-A-Lyzer dialysis device MWCO 1,000,000 (Fisher Scientific, Austria).

3.1.2 SEM Imaging

SEM images were obtained using ZEISS Supra 40 operating at 5 kV acceleration voltage (Carl Zeiss AG, Germany). The sample solutions were diluted with Milli-Q water. Then, 10 µl of the diluted sample solution was drop-casted onto a silicon wafer, which was left to dry at ambient conditions. Once the solvent has evaporated, the wafer was mounted onto a SEM sample holder equipped with a sticky, conductive tape, to ensure the correct placement. Template stripes were mounted onto a SEM sample holder.

3.1.3 PlasMag Measurements

The phase lag spectra were recorded with the PlasMag setup described in Schrittwieser *et al.*^{29,30}. All measurements were started within 5 min after mixing the sample solutions. Phase lag measurements were conducted at an external rotating magnetic field strength of 5 mT and discrete frequency values between 10 Hz and 1130 Hz. The software Origin

(OriginLab Corporation, USA) was employed for the graphical evaluation of the measured phase lag.

3.1.4 Zeta Potential Measurements

The particle size and zeta potential of nickel nanorods in aqueous solution were determined using Zetasizer Nano ZS (Malvern Instruments, UK). To that end, Milli-Q water with a resistivity of 18.2 M Ω cm was employed. Measurements of the zeta potential and the hydrodynamic size cannot be done straight forward with the software delivered by Malvern Instruments if nanorods instead of spherical particles are examined. While the zeta potential can be calculated by using the measured electrophoretic mobility and conductivity values as will be discussed later, all measured hydrodynamic size values have to be regarded as rough estimates. The underlying theoretical Smoluchowski model does not allow for quantitative determination of cylindrical particle geometry parameters. Nonetheless, it is possible to do a relative comparison of the measured size values.

3.2 Chemicals

The following chemicals and solvents were purchased from commercial sources and used without further purification: ultrapure aluminium foil (purity 99.999%, thickness 0.5 mm, 5 × 5 cm; GoodFellow, USA); sodium hydroxide pellets (NaOH), boric acid (H₃BO₃), oleic acid, nickel sulfate hexahydrate (NiSO₄·6 H₂O), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), poly(isobutylene-*alt*-maleic anhydride) (PMA) $M_w \approx 6000$, dodecylamine, tetrahydrofuran anhydrous (THF, 99.9 %), chloroform anhydrous (CHCl₃, 99 %) (Sigma Aldrich, Vienna); lauric acid, perchloric acid (HClO₄, 60 %), chromium trioxide (Cr₂O₃), ethanol (EtOH) (Merck, Vienna); nitric acid (HNO₃, 68 %), ortho-phosphoric acid (H₃PO₄, 85 %), sulfuric acid (H₂SO₄, 95-98 %) (Carl Roth, Karlsruhe); polyvinylpyrrolidone (PVP) K30 $M_w = 44000$ -54000 and oleic acid (Fluka, Vienna); polyvinylpyrrolidone (PVP) K12 $M_w = 3500$ (Acros Organics, Vienna), 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer anhydrous (Amresco, USA) and nickel chloride hexahydrate (NiCl₂·6 H₂O, Alfa Aesar, USA). The monoclonal antibody trastuzumab was kindly provided by the group of Prof. Dr. Frauke Alves at the Max Planck Institute for Experimental Medicine in Göttingen.

All solutions were prepared with Milli-Q water (resistivity of 18.2 M Ω cm), except stated otherwise.

3.3 General Procedures

3.3.1 Electrochemical Synthesis of Nickel Nanorods

3.3.1.1 Electropolishing the Aluminium Foil

Ultrapure aluminium foil was cleaned with isopropanol and acetone *via* sonication (5 min, room temperature, 45 kHz, 100 W). A cooling unit (140 mm height, 75 mm diameter), which served as an electrochemical cell, was filled with 440 ml HClO_4 -EtOH mixture (HClO_4 (60%)/EtOH 1:4) and cooled to 0 °C while stirring with a magnetic stir bar (50 mm length, 8 mm diameter). The stirring was stopped and the aluminium foil (anode) and a stainless steel sheet (cathode) were inserted into the electrochemical cell, as illustrated in Fig. 14. For 400 s, a voltage of 15 V was applied. Then, the template was removed from the electrochemical cell and rinsed with ethanol and Milli-Q water, dried with compressed air and stored in a petri dish equipped with fleece.

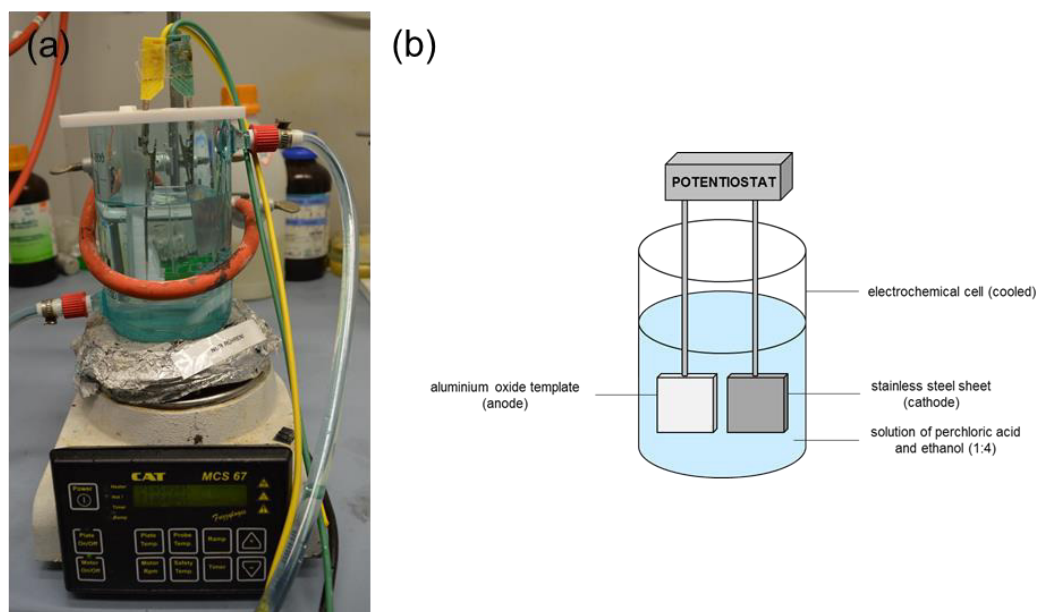


Figure 14 | Picture of the electropolishing setup (a) and its schematic illustration (b).

3.3.1.2 First Anodization of the Aluminium Template

The cell was filled with 440 ml H_2SO_4 (2 M) and cooled to 2 °C while stirring (200 rpm). The aluminium template (anode) and a platinum wire (cathode) were inserted into the electrochemical cell, as depicted in Fig. 15. Anodization was performed at a constant potential of 15 V for 18 hours by the Kepco power supply and a custom LabView program. After anodization, the voltage was automatically decreased to 5 V step by step (1 V steps, hold for 3 s). Then, the template was rinsed with Milli-Q water, dried with compressed air and then stored in a petri dish equipped with fleece.

The platinum wire was dipped into *aqua regia* (HCl conc./ HNO_3 conc. 3:1) after the anodization for 2 min and thoroughly rinsed with Milli-Q water.

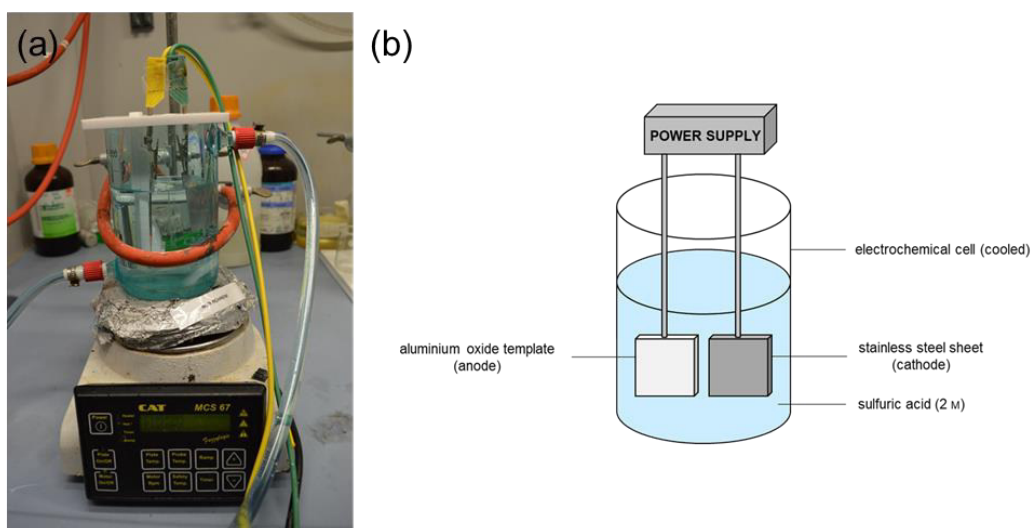


Figure 15 | Picture of the anodization setup (a) and its schematic illustration (b).

3.3.1.3 Dissolving of Aluminium Oxide

A 250 ml beaker glass was filled with 150 ml of a solution of H_2CrO_4 (0.2 M) and H_3PO_4 (0.6 M) (pH 2), as demonstrated in Fig. 16. The stirred (500 rpm) solution was heated to 60 °C in a heating block and then the template was kept in the solution for 4 h. Afterwards, the template was repeatedly rinsed with Milli-Q water and dried with compressed air. The edges and the backside of the template were then coated with three layers of nail polish (Manhattan Top Coat) and left to dry in the oven (60 °C).

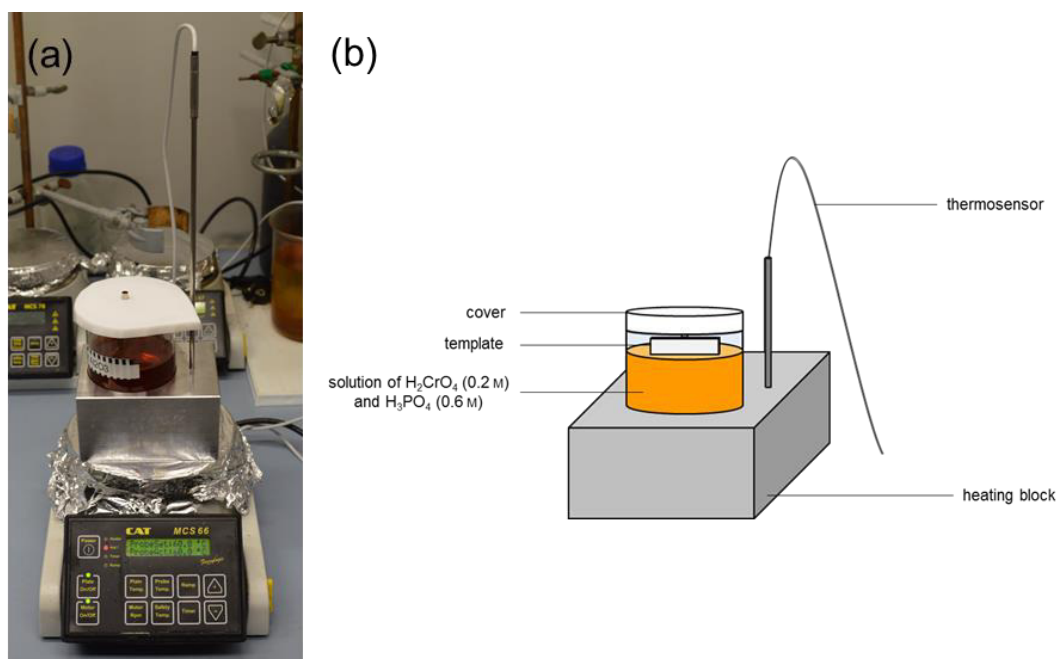


Figure 16 | Picture of the setup (a) and its schematic illustration (b).

3.3.1.4 Second Anodization of the Aluminium Oxide Template

The cell was filled with 440 ml H_2SO_4 (2 M) and cooled to 0 °C while stirring. The part of the aluminium oxide template, where an alligator clip would be fixed was roughened up with sandpaper to allow for proper electrical connection. Then, the aluminium template (anode) and a platinum wire (cathode) were inserted into the electrochemical cell, as depicted in Fig. 15, and the anodization was started. The anodization process stopped automatically after the flow of an overall charge quantity of 2 C/cm² at a potential of 14 V, resulting in a pore diameter of 13 nm. After anodization, the voltage was decreased step by step (1 V steps, hold for 3 s) and the aluminium oxide template was rinsed with Milli-Q water and immediately immersed into H_3PO_4 (0.1 M) for the following step.

3.3.1.5 Homogenization of Pores

The aluminium oxide template was immersed into a 250 ml beaker filled with 100 ml H_3PO_4 (0.1 M) and shaken repeatedly (room temperature, 5 min) to homogenize the inner layer of the pore channels and to thin out the barrier oxide layer. Afterwards, the template was rinsed with Milli-Q water and stored in Milli-Q water to prevent desiccation of the pore channels for the subsequent electrodeposition step.

3.3.1.6 Electrodeposition of Nickel into Pores

The stirred Nickel-Watts bath was heated to 80 °C until the precipitates dissolved. Then, a 250 ml beaker was filled with 150 ml Nickel-Watts solution and cooled to 35 °C in a water bath, while stirring. A constant current density of 50 mA/cm² was set and the timeframes for the pulsed deposition were selected as $t(-) = 16$ ms, $t(+) = 2$ ms and $t(0) = 200$ ms on the Autolab potentiostat. Prior to the actual nickel deposition, a copper wire was inserted into the electrochemical cell as cathode (working electrode, WE), a nickel wire as anode (counter electrode, CE) and Ag/AgCl electrode as reference electrode (RE) and 5 electrodeposition cycles were performed. This procedure was repeated once more to ensure correct initialization of the potentiostat. Then, the aluminium oxide template was inserted into the cell as cathode (WE), as illustrated in Fig. 17, instead of the copper wire and left there for at least 10 min to guarantee diffusion of metal ions from the solution into the pore channels. Subsequently, 150 electrodeposition cycles were performed and the template was thoroughly rinsed with Milli-Q water, dried with compressed air and then stored in a petri dish equipped with fleece.

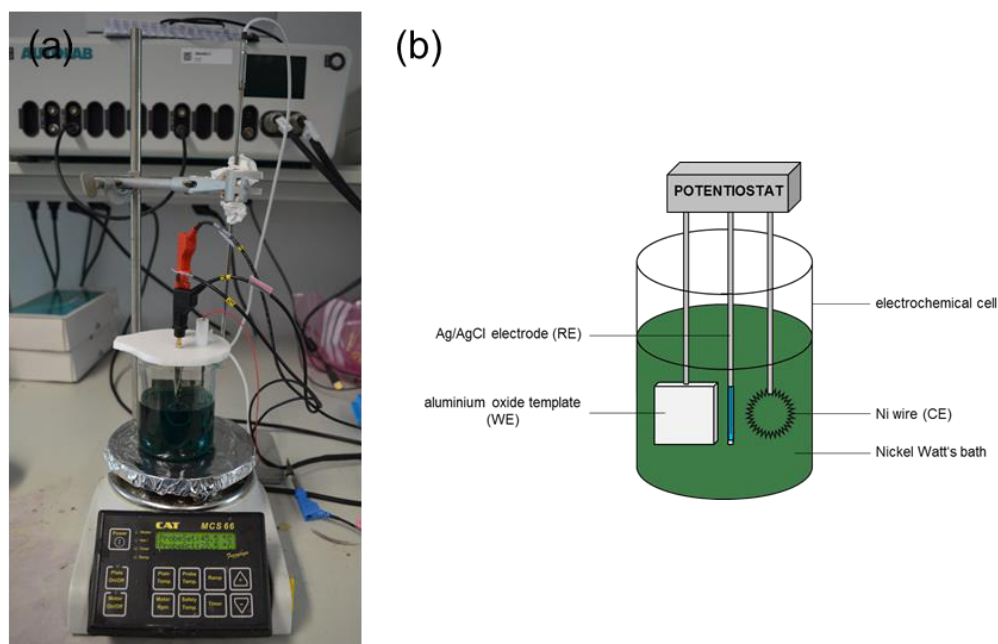


Figure 17 | Picture of the electrodeposition setup (a) and its schematic illustration (b).

3.3.1.7 Extraction of Nickel Nanorods from Aluminium Oxide Template and Transfer into Colloidal Suspension

The nail polish on the backside was removed by scratching with a pin needle and peeling off the nail polish layer. Next, the aluminium template was cut into four pieces, which were then each immersed in 3 ml NaOH (0.1 M) in a 4 ml screw-cap vial. Then, the samples were sonicated (20 min, 20 °C, 45 kHz, 100 W) and the template strips were removed from the clear solution with black/grey nanoparticle-agglomerates. Three magnets were attached to each vial and the samples were left to settle overnight. The supernatant was discarded with a syringe and replaced with 3 ml NaOH (0.1 M). The particles were left to settle for approximately 30 min and then the washing step was repeated two more times. Subsequently, the supernatant was discarded again, replaced with 3 ml Milli-Q water or EtOH, depending on the following functionalization approach. The washing step was repeated analogously two more times. After the last washing step, the particles were dispersed in 1 ml Milli-Q water or EtOH, again depending on the functionalization approach.

3.3.2 Stabilization of Colloidal Nanorod Dispersions

The appropriate amounts of surfactant and PMA was calculated using the equations given in the appendix.

3.3.2.1 Nanoparticle coating with fatty acids

The nanorod sample dispersion was sonicated (30 min, 20 °C, 45 kHz, 100 W) and the desired amount of oleic acid, lauric acid or PVP was dissolved in 1 ml EtOH. To 1 ml sample dispersion in EtOH, 1 ml surfactant solution was added and the mixture was sonicated (140 min, 20 °C, 45 kHz, 100 W). The water of the ultrasonic bath was exchanged regularly to maintain water bath temperatures of 20-30 °C. Then, the samples were incubated on a shaker (24 h, room temperature) and sonicated (30 min, 20 °C, 45 kHz, 100 W), prior to PlasMag measurements.

3.3.2.2 Polymer synthesis of dodecylamine modified PMA

According to the protocol of Lin *et al.*¹¹⁴, 0.77 g PMA (M_w 6000) was vigorously mixed with dodecylamine in 25 ml anhydrous THF. The opaque solution became transparent upon heating at 60 °C for approximately 20 min under vigorous magnetic stirring. The reaction mixture was refluxed overnight and was then cooled to room temperature. After evaporation of the organic solvent under reduced pressure in a rotary evaporator (20 min, 60 °C, 400 mbar, 120 rpm, then 20 min, 70 °C, 55 mbar, 120 rpm), 15 ml anhydrous THF was added and the solution was evaporation under reduced pressure was repeated. Finally,

the residue was dissolved in 15 ml anhydrous chloroform. The final polymer solution showed a slightly yellow color.

3.3.2.3 Nanoparticle coating with PMA

For the coating with the amphiphilic polymer PMA, the nanorod sample dispersion was sonicated (20 min, 20 °C, 45 kHz, 100 W) and the appropriate volume of PMA solution was added to 1 ml nanoparticle dispersion in EtOH. This mixture was sonicated (30 min, 20 °C, 45 kHz, 100 W) and evaporated in a rotating evaporator under reduced pressure (20 min, 50 °C, 100 mbar, 150 rpm). The dried polymer-particle solution was redissolved in 1 ml EtOH, sonicated (30 min, 30 °C, 45 kHz, 100 W) and evaporated in a rotating evaporator (20 min, 50 °C, 100 mbar, 150 rpm) to ensure that the PMA is well wrapped around the nanorods. Then, the dried solution was redissolved in 1 ml NaOH (0.01 M) to hydrolyze the polymer backbone consisting of succinic anhydride groups. After sonication (30 min, 20 °C, 45 kHz, 100 W) to disperse all particles, phase lag spectra were recorded by PlasMag measurements. Next, the particles were magnetically separated and washed with Milli-Q water three times and the phase lag measurements were repeated.

4 Results and Discussion

4.1 Electrochemical Synthesis of Nickel Nanorods

4.1.1 Electropolishing the Aluminium Foil

The electropolishing of the aluminium template yielded a smooth, mirror-like surface, compared to a rough, unpolished template, as can be seen in Fig. 18. In the following graphs, the current is denoted as a negative value, due to the wiring of the employed multimeter. It can also be regarded as an absolute value under consideration that the template represents the anode in the electrical circuit. In contrast to the anodization process, where diluted sulfuric acid is employed to achieve pore formation, a mixture of ethanol and perchloric acid is used as electrolyte. Since the dissolving of the oxide takes place faster than the formation in this case, the removal of material is favored.

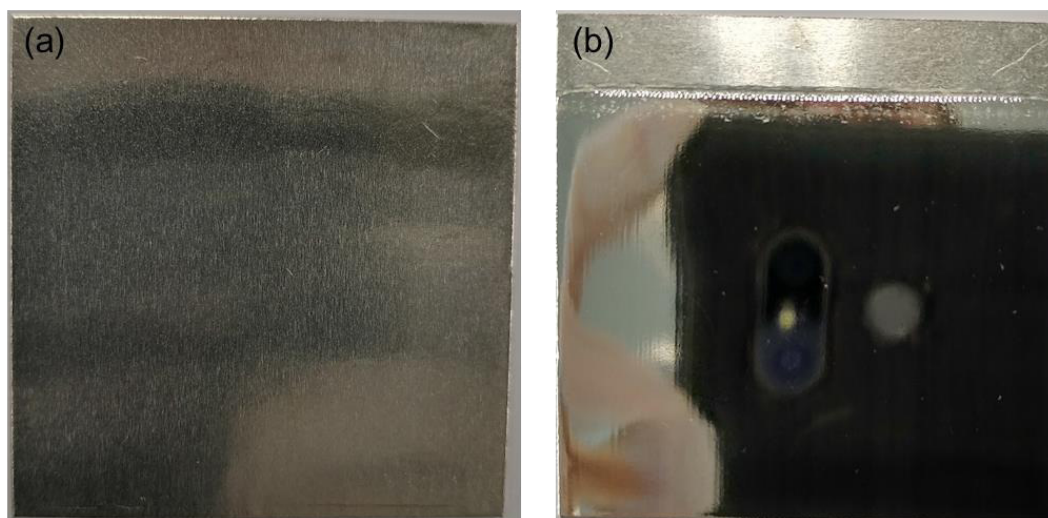


Figure 18 | Picture of a commercially available aluminium template before (a) and after (b) electropolishing. In picture (b), the reflexion of the phone, used for taking the pictures, is clearly visible in contrast to picture (a), which is due to the reduced surface roughness of the electropolished template.

The electric field lines focus on inhomogeneities on the template surface and lead to enhanced polishing at protruding surface areas. As the electropolishing process progresses, the surface gets smoother gradually and the interface template-electrolyte decreases simultaneously, hence the fast decrease in current, as demonstrated in Fig. 19.

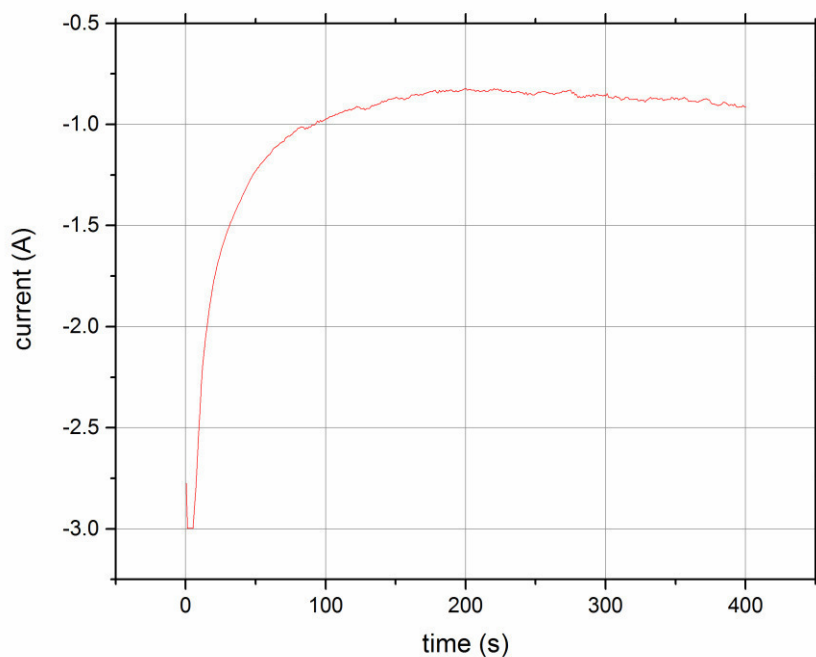


Figure 19 | Current characteristics during the electropolishing process.

Due to the range of the multimeter, the current is limited to a maximum of 3 A. The smoothness of the surface increases with the duration of the electropolishing until a certain threshold is reached and the current remains nearly constant. Therefore, the electropolishing process was carried out, until the current reached a plateau, which happened after 400 s. If the electropolishing is performed longer than necessary (*e.g.* 800 s) or with thinner templates (*e.g.* thickness of 0.25 mm), the templates becomes wafer-thin and brittle. Hence, the handling of the template and subsequent steps are increasingly difficult to perform. Undesired, irregular cracks in the micro to nanometer scale are produced, which are then filled with nickel during the electrodeposition, thereby causing contamination of the nanorod solutions, as can be seen in Fig. 20, since they should only contain uniformly shaped particles.

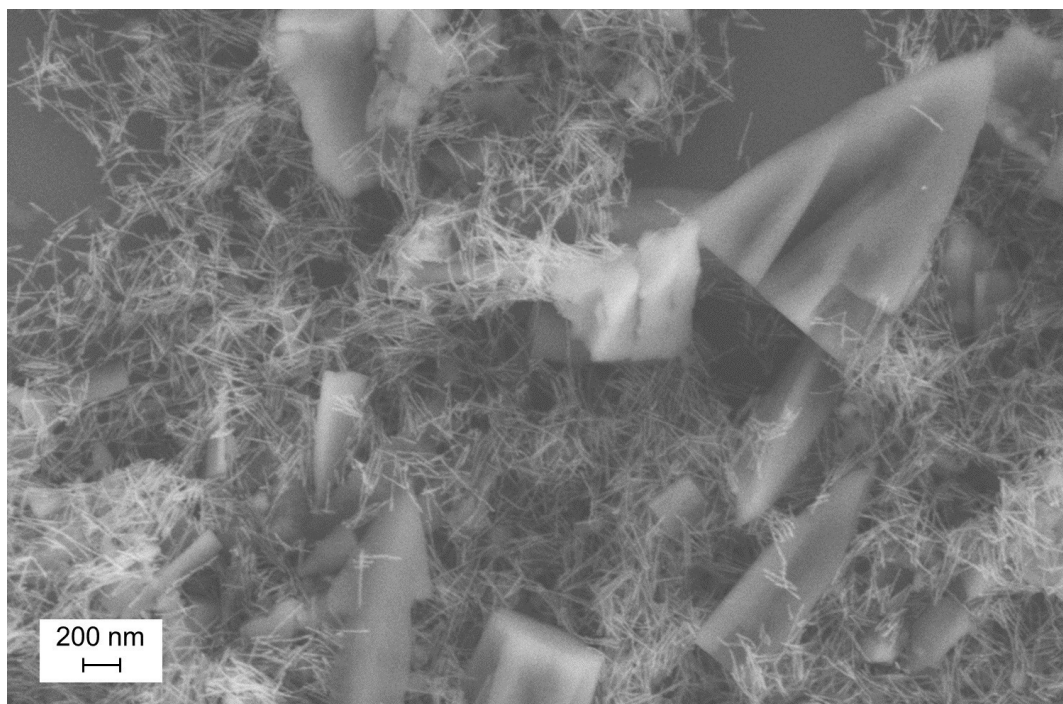


Figure 20 | SEM image of a nickel nanorod solution contaminated with randomly shaped nickel particles with sizes in the nano- to micrometer range.

4.1.2 First Anodization of the Aluminium Template

When the voltage is first applied, only the native aluminium oxide layer is present. Due to the low resistance, the current reaches a maximum, as can be seen in segment A of Fig. 21.

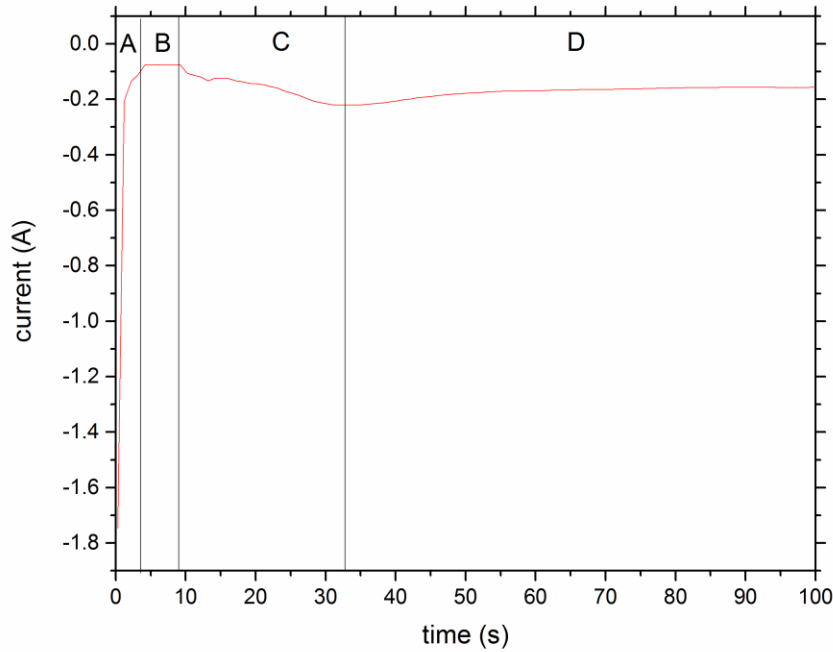


Figure 21 | Current characteristics for the first 100 s of the first anodization process. The segments A-D are described in the text.

Through the formation of a thicker oxide layer, the so-called barrier oxide, the resistance increases and causes a decrease in current. The electric field lines focus in slots of the inhomogeneous and rough surface of the barrier oxide and lead to enhanced electrolysis of water molecules as well as dissolving of the oxide in slightly corrosive electrolytes like sulfuric acid. As demonstrated in segment B, the current reaches a minimum and stays mostly constant then, as cavities on the surface of the barrier are formed. Since the electric field lines remain focused at the bottom of the cavities, the dissolution of the oxide resumes and a decrease in resistance as well as an increase in current result, while the formation of pores is initiated (see segment C). Due to the dissolving process, the barrier oxide is thinned down and as soon as ions can drift through, formation and dissolving of oxide proceed simultaneously and an equilibrium is established. Therefore, a constant thickness of the barrier oxide is reached and pores are steadily formed, hence no changes in current, as can be seen in segment D and in Fig. 22.

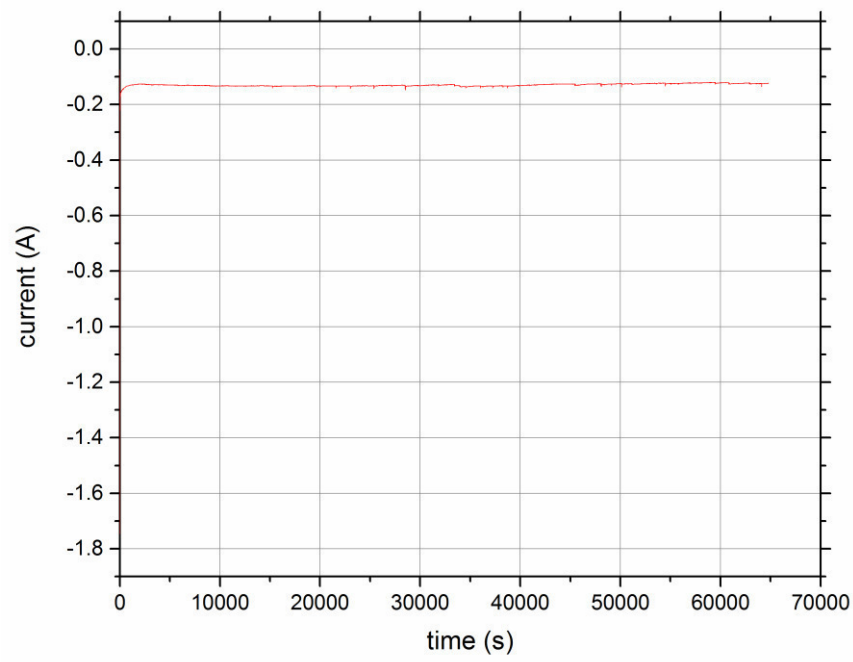


Figure 22 | Current characteristic of the first anodization process.

4.1.3 Second Anodization of the Aluminium Oxide Template

Prior to the second anodization of the template, the aluminium oxide layer is chemically selective dissolved by a mixture of chromic acid (0.2 M) and phosphoric acid (0.6 M), since the pores are still randomly ordered and the pore channels are inhomogeneous. The resulting pore imprint is then employed for the second anodization. In contrast to the first anodization, the formation of pores starts at the hexagonally ordered pore imprint instead of random cavities across the aluminium oxide template. Hence, an equilibrium between formation and dissolving of the oxide is established faster than in the first anodization, which ultimately leads to less significant changes in current, as can be seen in Fig. 21 compared to Fig. 23.

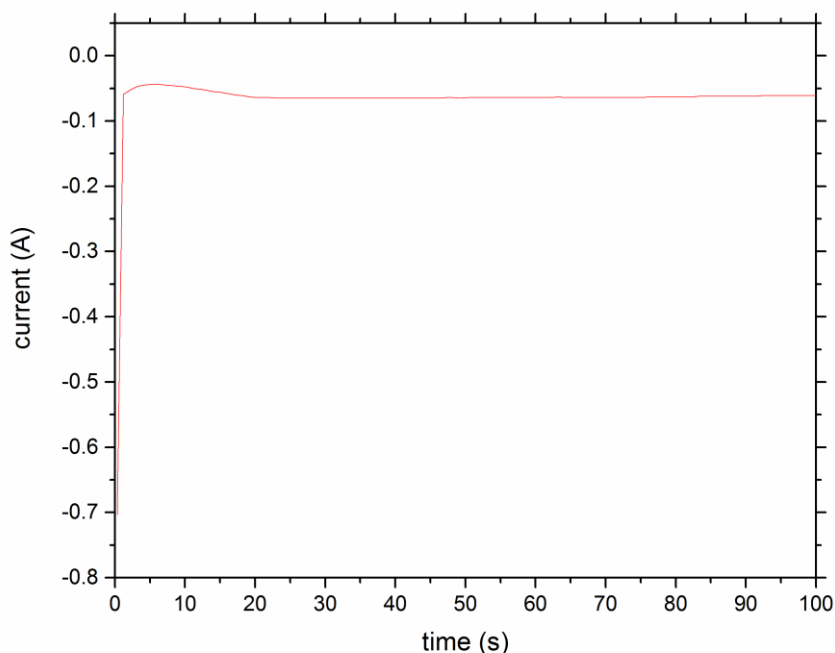


Figure 23 | Current characteristics for the first 100 s of the second anodization process.

Furthermore, the second anodization ends as soon as a flow of overall charge quantity of 2 C/cm^2 is reached, to achieve uniform pore growth of approximately 800 nm pore length, since the thickness of the oxide layer can be controlled by adapting the flow of overall charge quantity. Commonly, the process is finished after 10-12 minutes, as can be seen in Fig. 24.

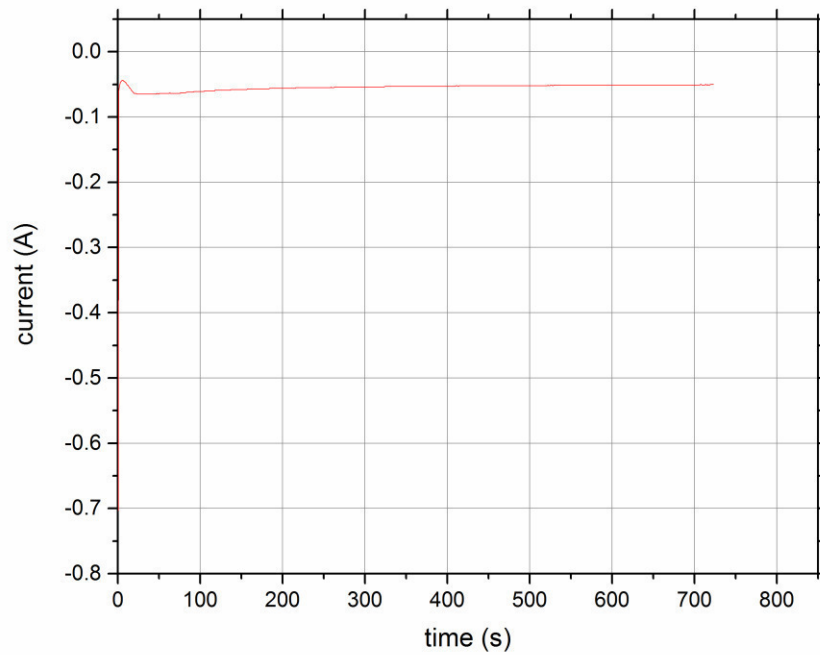


Figure 24 | Current characteristic of the second anodization process.

The desired pore diameter can be controlled by adapting the applied voltage. For an intended pore diameter of 13 nm, a voltage of 14 V was applied. As demonstrated in Fig. 25, the second anodization leads to an aluminium oxide template with highly ordered, hexagonal pore structure.

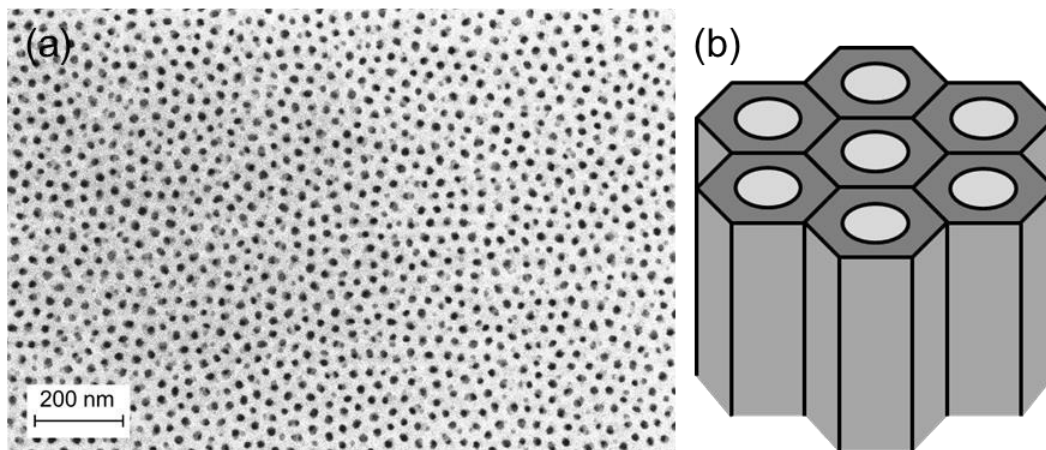


Figure 25 | (a) SEM image of the highly ordered, hexagonal pore structure of the aluminium template after the second anodization. (b) Schematic illustration of the hexagonal pore structure.

4.1.4 Electrodeposition of Nickel into Pores

Electrodeposition of nickel into the pores of the aluminium oxide template was achieved by applying short, constant current pulses, consisting of a negative pulse of 16 ms and a positive pulse of 2 ms, followed by a longer pauses of 200 ms, as illustrated in Fig. 26.

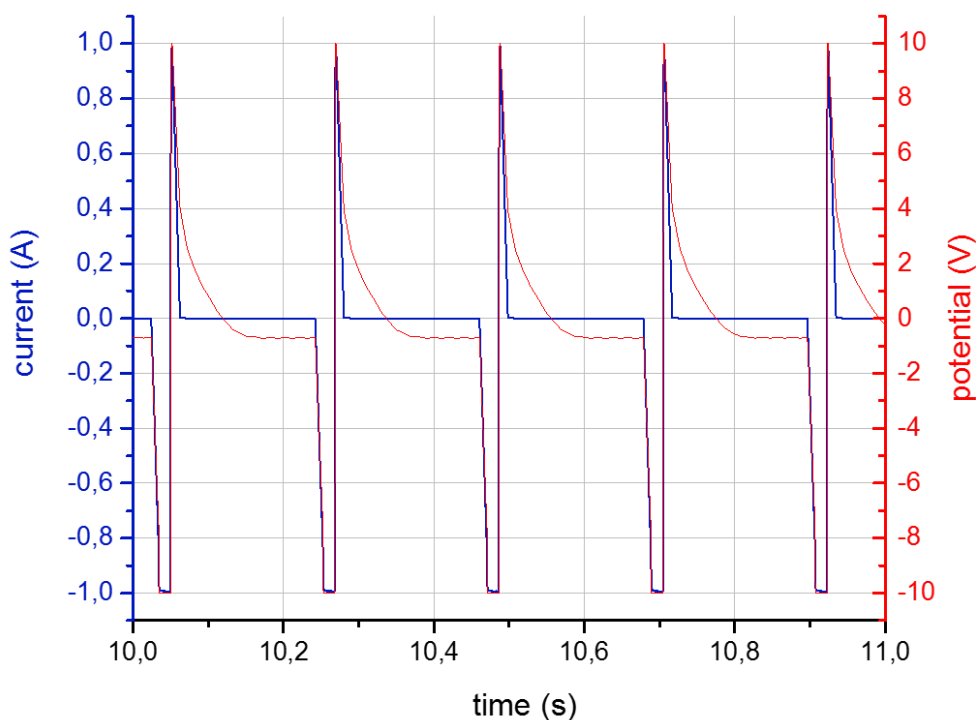


Figure 26 | Current-voltage characteristics of the electrodeposition process.

During the deposition process, the bottom of the pores is depleted of Ni^{2+} ions and a concentration gradient inside the pores is formed. By adjusting the pause period, the rate of deposition can be controlled to ensure sufficient ion transportation by thermic diffusion through the pore channels. After 150 pulse cycles, consisting of a negative and a positive pulse, as well as the pause period, nanorods with lengths of approximately 200 nm and a diameter of approximately 15 nm were obtained, as confirmed by SEM images. Successful electrodeposition is indicated by a change of color of the aluminium oxide template from silver to red or green (depending on the angle of vision), as can be seen in Fig. 27.

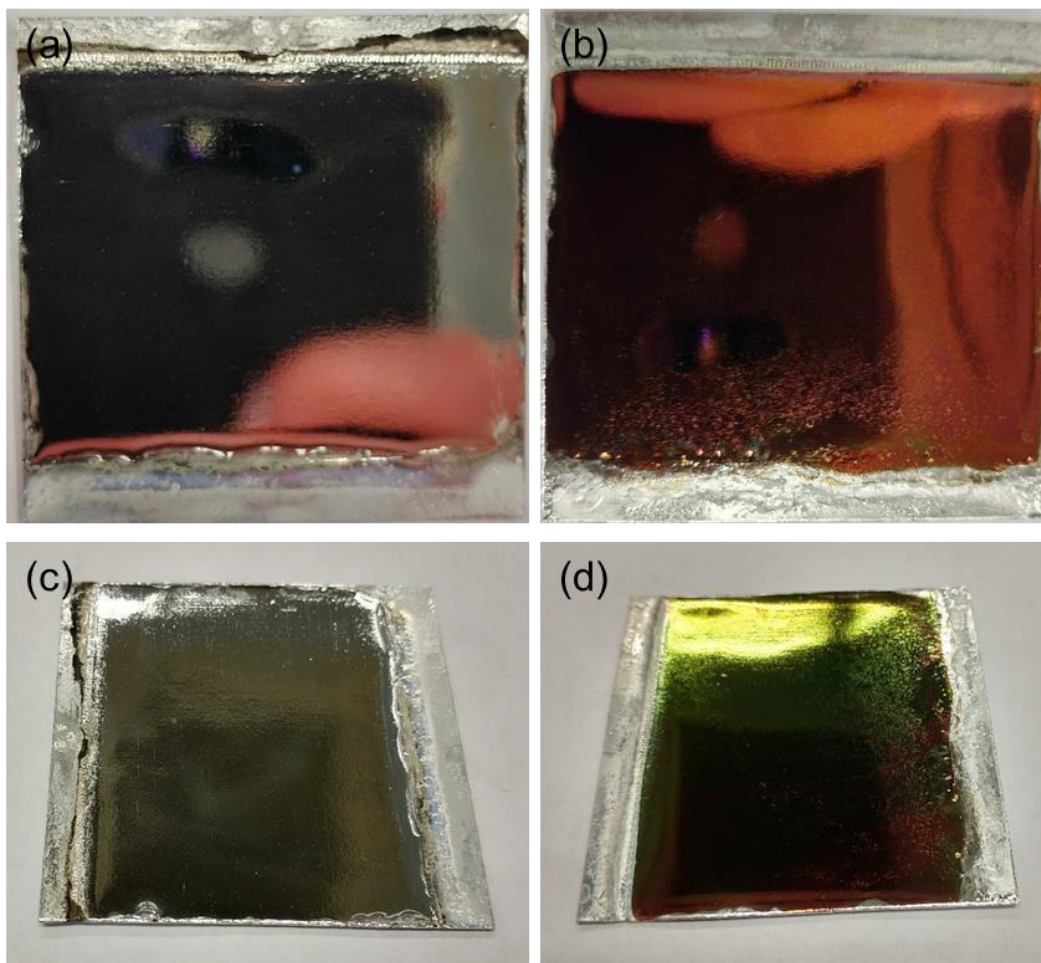


Figure 27 | Aluminium template before (a), (c) and after (b), (d) electrodeposition.

In Fig. 28 and Fig. 29, the template was cut and analyzed using electron microscopy after electrodeposition. Although it is difficult to give a more detailed interpretation of Fig. 28 due to the distortions caused by the mechanical cutting, it reveals the ordered, hexagonal pore structure of the aluminium oxide template. Fig. 29 demonstrates nanorods and nanorod segments still embedded in the pore channels, but broken into pieces due to the blunt force of cutting.

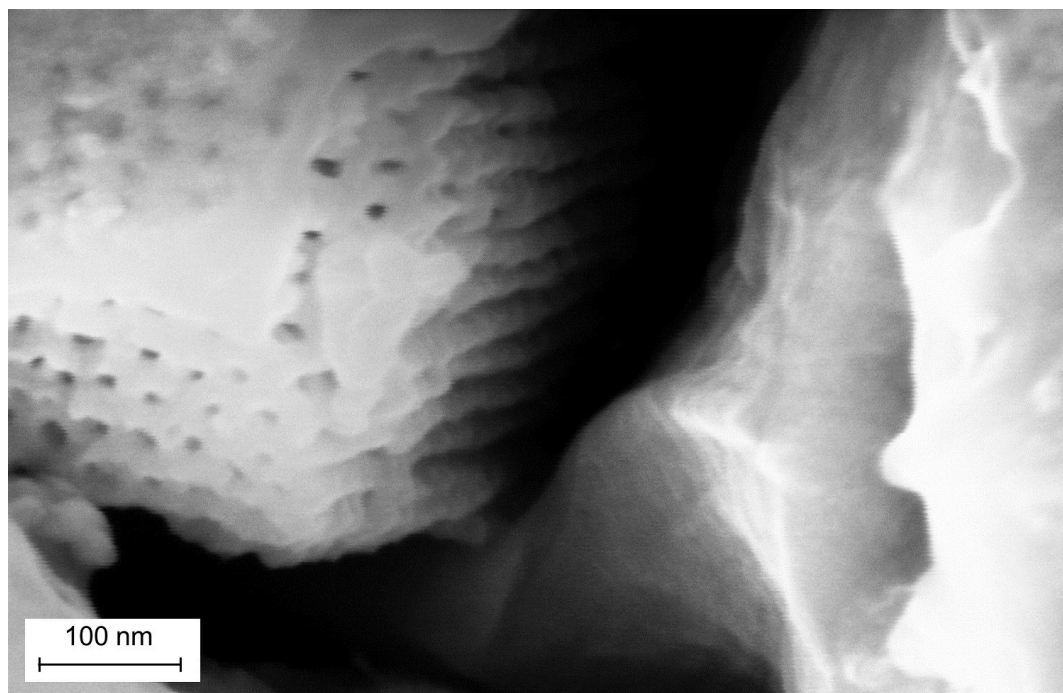


Figure 28 | SEM image of the ordered, hexagonal pore structure of the aluminium oxide template after electrodeposition. Due to the mechanical cutting, the template is distorted.

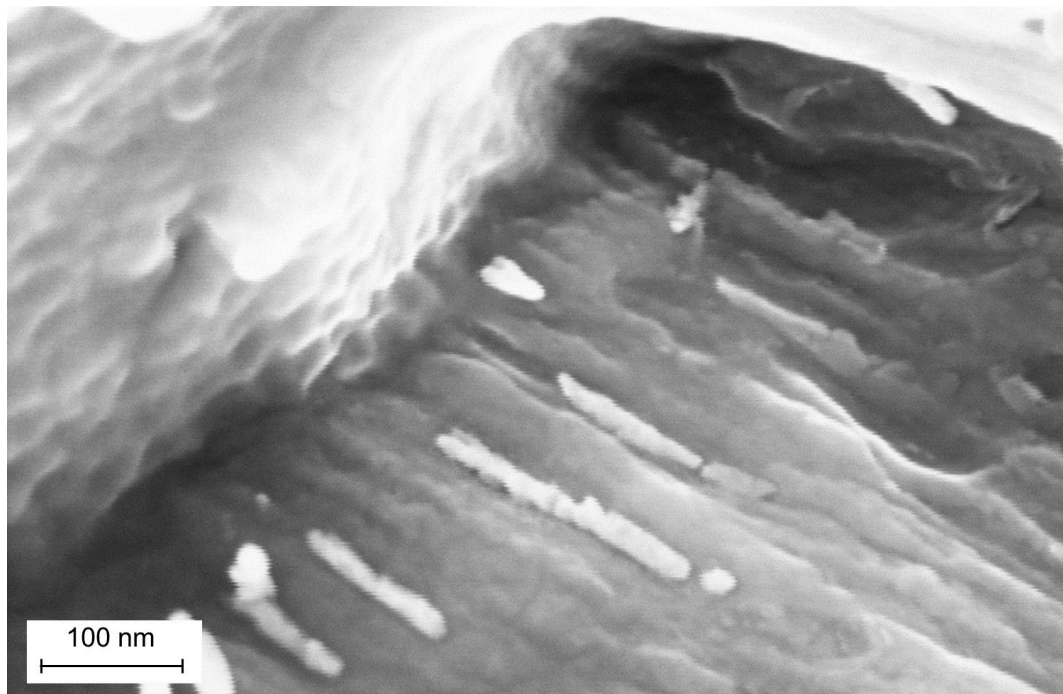


Figure 29 | SEM image of nickel nanorods in the pore channels of the aluminium oxide template. The particles are shorter and partially segmented due to the mechanical cutting.

4.2 Stabilization of Colloidal Nanorod Dispersions

By stabilizing the nanoparticles, agglomeration by attracting van der Waal's forces and magnetic dipole-dipole forces can be prevented, as schematically depicted in Fig. 30. For this reason, stabilization approaches by electrostatic, steric or electrosteric repulsion are employed to modify the surface of nanoparticles.

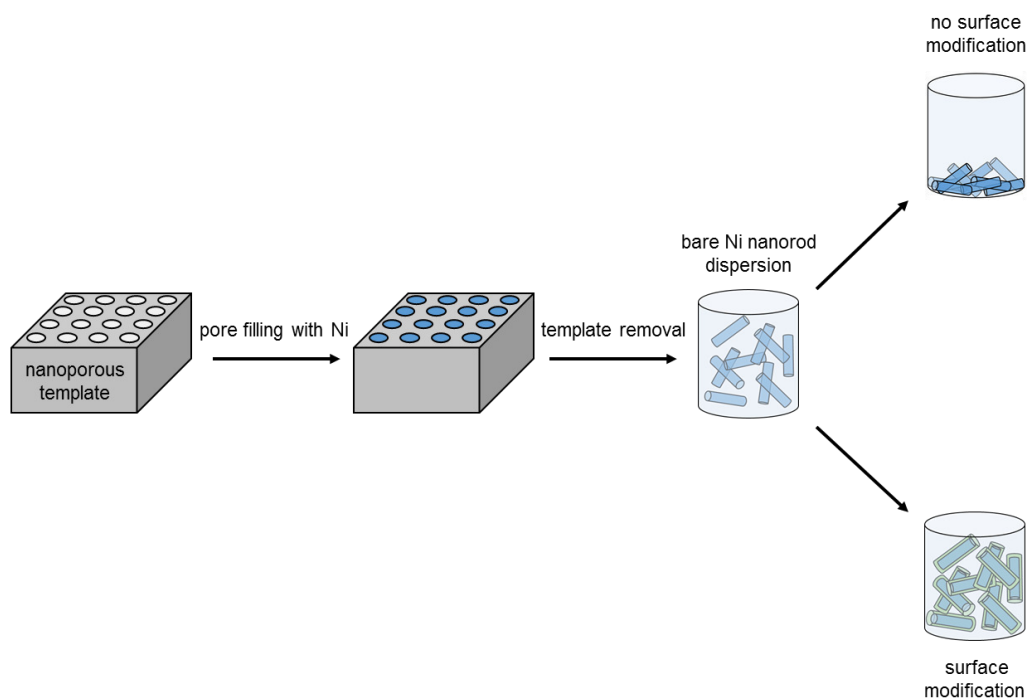


Figure 30 | Schematic illustration of the synthesis procedure of nickel nanorod dispersions. In a first step, nickel is electrodeposited into a nanoporous template. Then, the template is chemically selective dissolved, which results in a dispersion of bare nickel nanoparticles. Finally, the surface of the nickel nanoparticles is modified to prevent agglomeration and to obtain stable dispersions.

Nanoparticles with unmodified surface chemistry tend to agglomerate and are not stable in colloidal dispersion. For this reason, the surfactants depicted in Fig.31 are added to the nanorod dispersions either during the extraction process or afterwards.

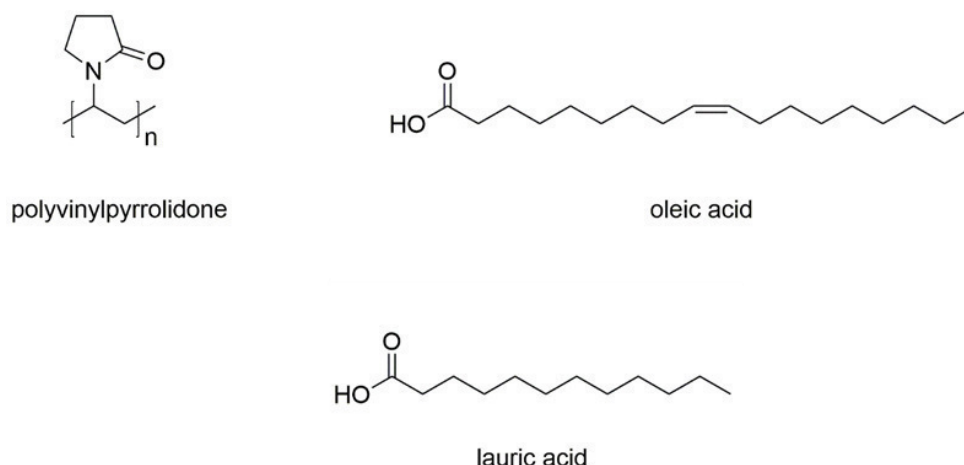


Figure 31 | Chemical structures of the molecules employed for nickel nanorod surface modification.

The high affinity of carboxylic acids to the native nickel oxide layer provides theoretical basis for stabilization approaches with fatty acids like oleic acid and lauric acid. Wu *et al.*¹¹⁵ describe the chemisorption of oleic acid as a carboxylate onto cobalt (Co) nanoparticle surfaces as a carboxylate, where the two oxygen atoms are coordinated symmetrically to the cobalt atoms, leading to the formation of covalent Co-O bonds. Based on the results of Timmons and Zisman¹¹⁶, it can be assumed, that fatty acids adsorb to the nickel nanoparticle surface analogously, as illustrated in Fig. 32. The particles are coated with fatty acids to produce a hydrophobic layer, which is then further used as a foundation for the coating with poly(isobutylene-*alt*-maleic anhydride) (PMA) modified with dodecylamine. Succinic anhydride within the PMA polymer is hydrolyzed with aqueous sodium hydroxide to yield carboxy groups. These carboxy groups are then partially modified with dodecylamine, resulting in the formation of amidated PMA and providing the hydrophobic regions for the subsequent self-assembly process, whereas unmodified carboxy groups provide reactivity for the antibody conjugation through EDC/sulfo-NHS linker chemistry. Here, the hydrophobic regions of the amidated PMA align with the fatty acid alkyl chains in a self-assembly process driven by hydrophobic van der Waals interactions, thereby creating the coating layer made from PMA polymer.

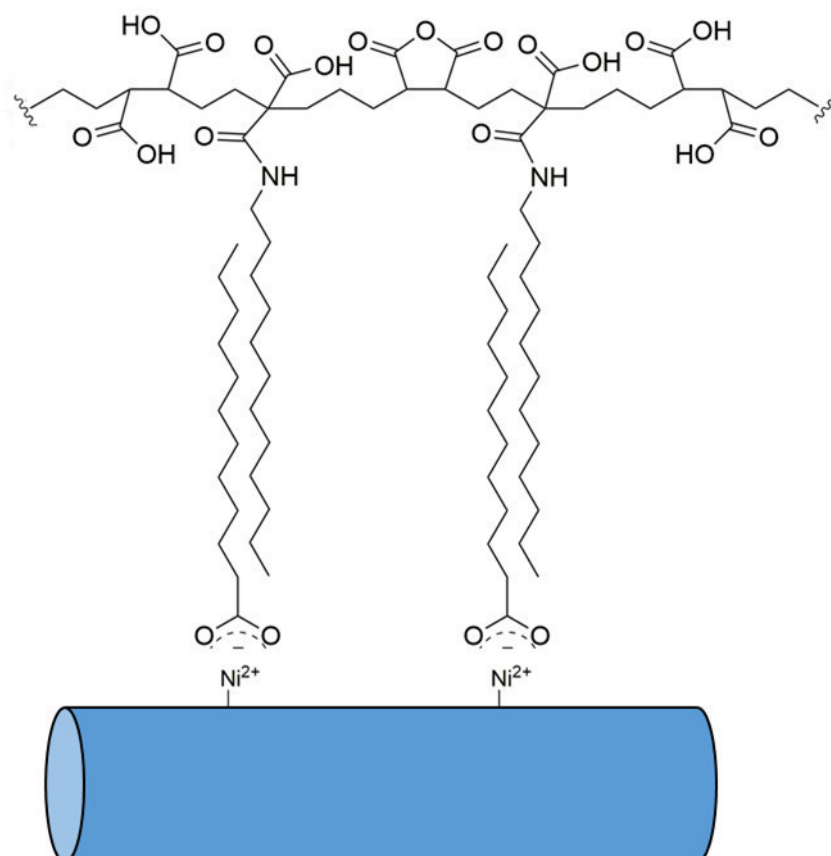


Figure 32 | Schematic illustration of a nickel nanorod coated with lauric acid and PMA modified with dodecylamine after hydrolysis. The polymer is partially hydrolyzed yielding carboxy groups next to succinic anhydride groups and *N*-dodecyl-acetamide groups.

4.2.1 SEM Imaging

The Fig. 33-Fig. 35 show nanorods during different stages of the extraction and coating process. In Fig. 33, the template was immersed in NaOH (0.1 M) to dissolve the aluminium oxide and to set the nickel nanorods free. Fig. 34 shows bare, uncoated nanoparticles after the extraction procedure and several washing steps, while washed, PVP-coated nanoparticles are illustrated in Fig. 35.

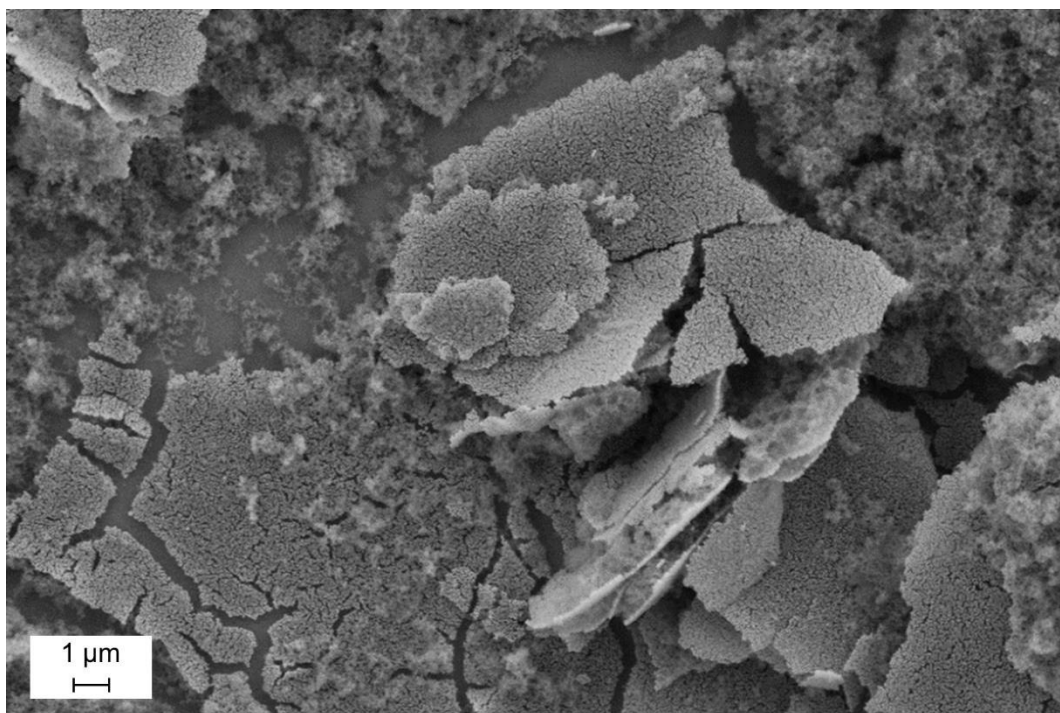


Figure 33 | SEM image of nickel nanorods partially embedded in the aluminium oxide template during the extraction process.

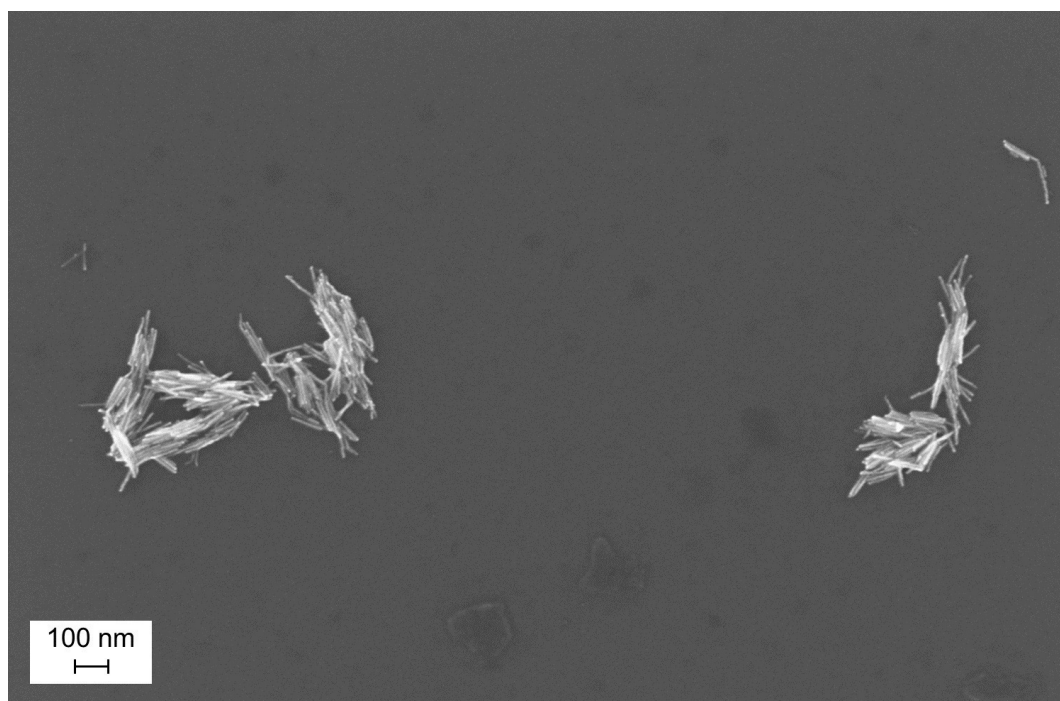


Figure 34 | SEM image of nickel nanorods after the extraction process and several washing steps.

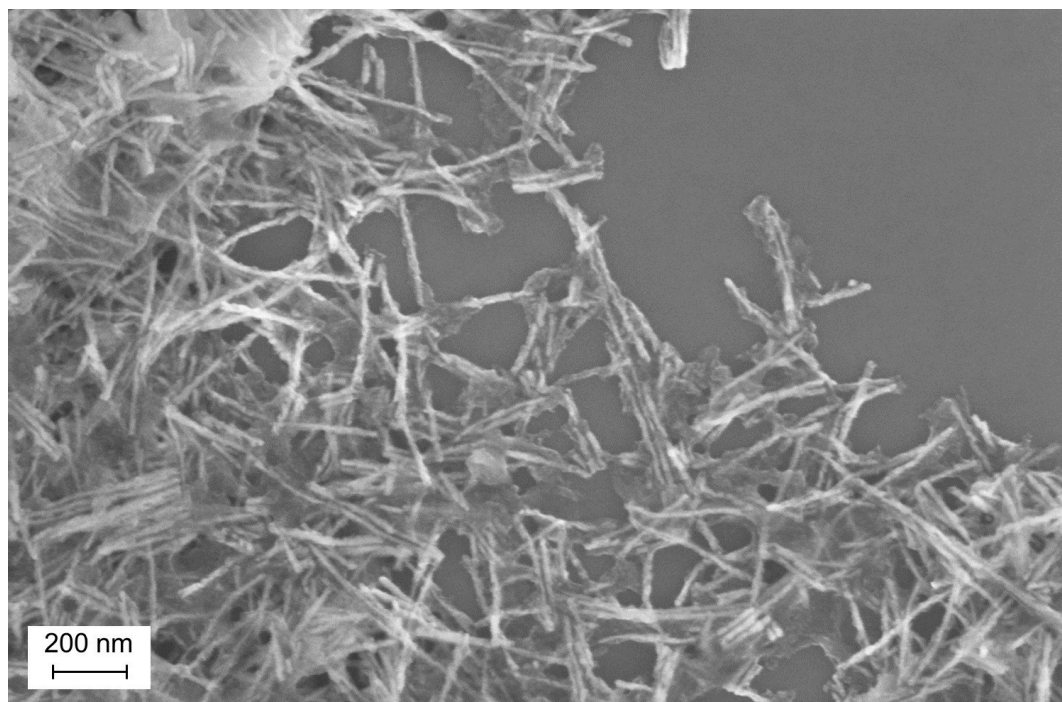


Figure 35 | SEM image of nickel nanorods coated with PVP.

While the particles are still ordered in the template in Fig. 33, they adapt a random order as soon as the aluminum oxide template is dissolved, which can be seen in Fig. 34 and Fig. 35. In contrast to the bare particles, the particles in Fig. 35 are covered in a polymer layer, due to the addition of PVP during the extraction.

The average nanorod length of $201.5 \text{ nm} \pm 13.6 \text{ nm}$ was determined by manually measuring the size of 30 nanorods from a SEM image. A diameter of approximately 15 nm was also determined from SEM images. In Fig. 36, the length distribution of nanorods is represented in a histogram.

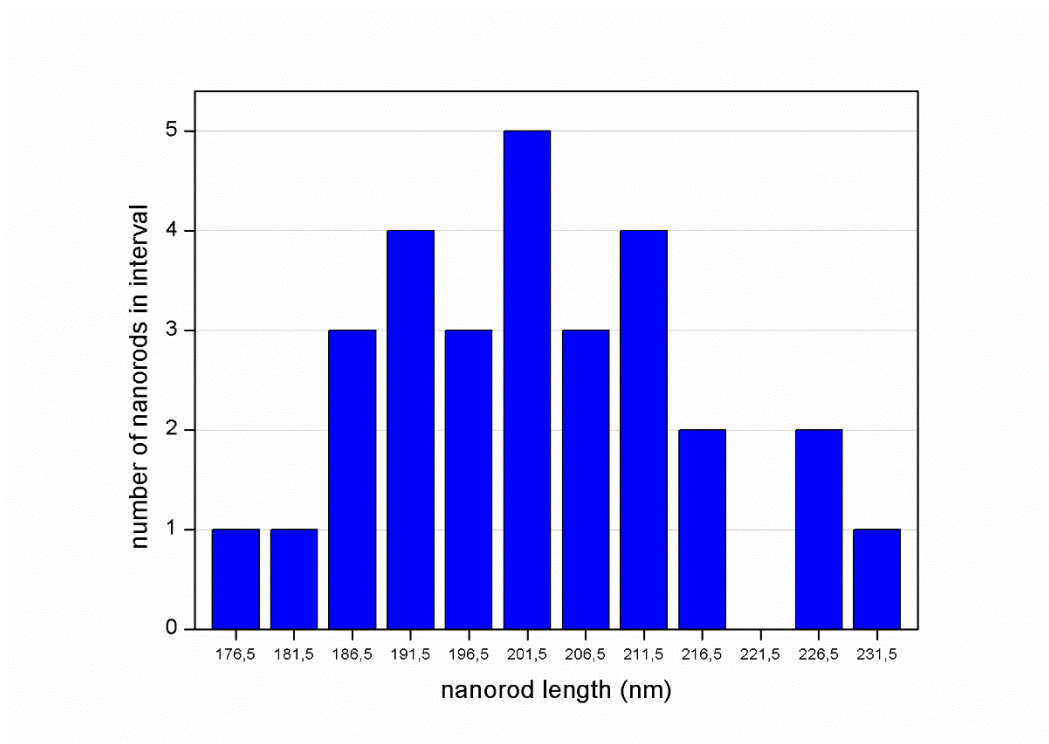


Figure 36 | Histogram of the nanorod size distribution (n=30).

4.2.2 Zeta Potential Measurements

The water-soluble polymer PVP adsorbs to the surface of the nickel nanorods through its hydrophilic pyrrolidone group. Due to the polymer layer with varying chain lengths, steric repulsions prevent agglomeration of particles, which was also confirmed by zeta potential measurements, as can be seen in Fig. 37. A zeta potential of 35.00 mV was determined for PVP-coated nanoparticles, which is slightly lower but well comparable to the zeta potential determined by Schrittwieser *et al.*¹¹⁷. Thus, this result indicates, that the stabilization through addition of PVP is achieved by electrosteric repulsion. Along with the SEM image shown in Fig. 35, this finding also suggests, that the nanoparticle surface is altered due to the addition of PVP during the extraction process.

The positive zeta potential value for PVP-coated nanoparticles is reasonable, because a nickel oxide layer is formed on the surface of nickel nanorods and nickel oxide has a point of zero charge above the pH of the sample solution, as previously described by Schrittwieser *et al.*¹¹⁷. Since the Smoluchowski model¹¹⁸ cannot be used for cylindrical nanoparticles, the measured zeta potentials were corrected as described by Schrittwieser *et al.*³⁰.

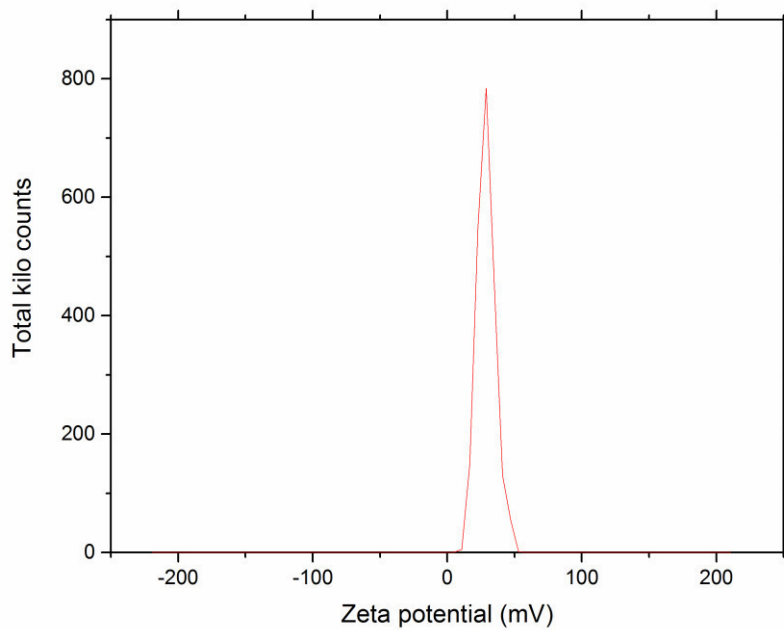


Figure 37 | Zeta potential of nickel nanorods coated with PVP.

The measured negative zeta potential of -88.46 mV for PMA-coated particles can be explained by the negative charge of the unmodified carboxy groups of PMA, which were created during the hydrolysis of succinic anhydride. The highly negative zeta potential indicates high stability of the aqueous nanoparticle dispersion, which allows subsequent antibody conjugation of these particles.

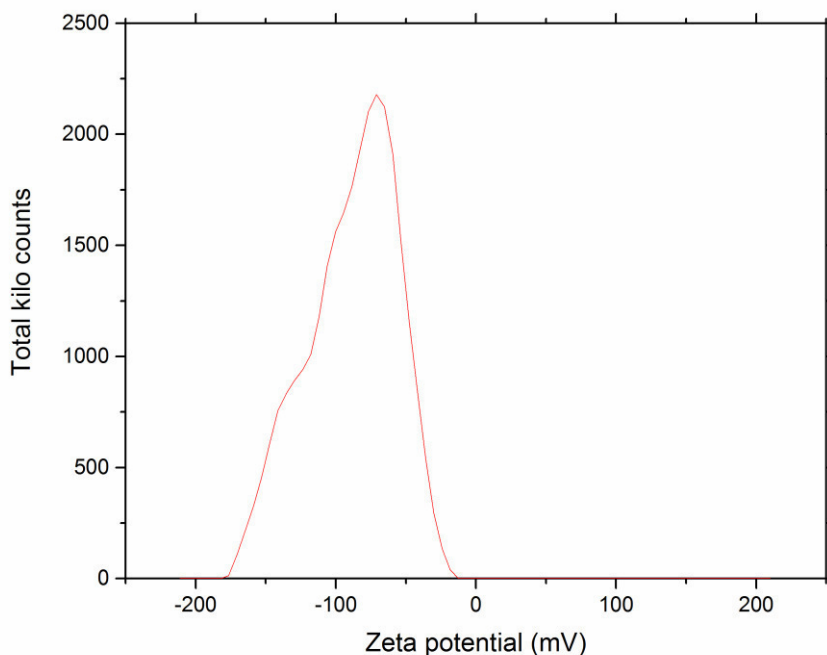


Figure 38 | Zeta potential of nickel nanorods coated with oleic acid and modified PMA.

The zeta potential measurements along with the hydrodynamic size measurements for the different stabilization approaches are summarized in Table 1 and Fig. 39. The zeta potential measurements are supported by the measurements of the hydrodynamic nanorod size. The measured similar size values for PVP and PMA coated nanorods indicate that no particle agglomeration effects were detected. Furthermore, it can be followed that the measured zeta potential values originate from the same source. It can be assumed that this source are the nickel nanorods and not any side product stemming from the polymer coating process (*e.g.* polymer micelles without nanorod involvement).

Table 1 | Zeta potential and size measurements of differently coated nickel nanoparticles.

	Zeta potential / mV	Number of Zeta runs	Size ¹ / nm	PDI
PVP-coated NP	35.00	22	208.5	0.276
PMA-coated NP	-88.46	200	207.6	0.227

¹ The results of the size measurements must be interpreted as rough approximations of the true values, since the Smoluchowski model only accounts for spherical particles.

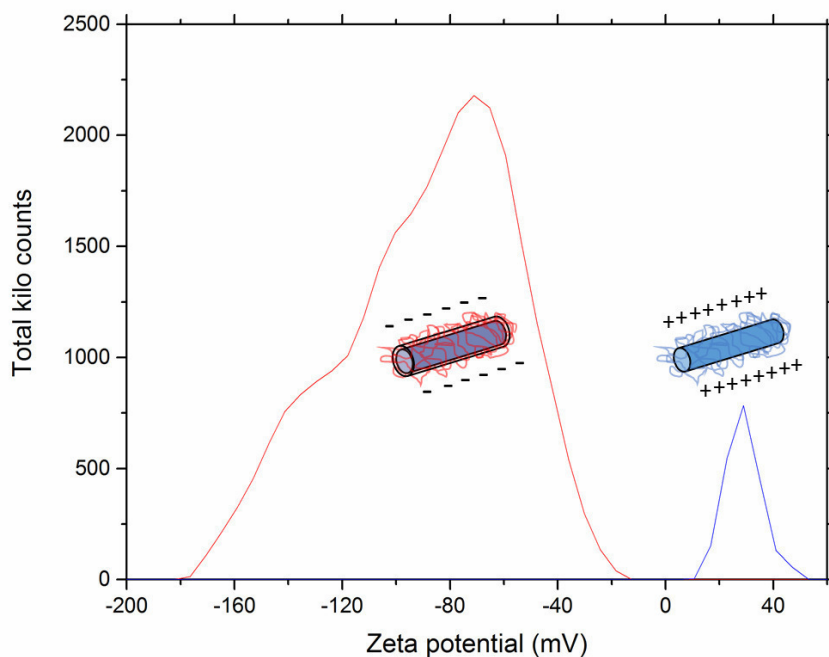


Figure 39 | Zeta potential of nickel nanorods coated with oleic acid and modified PMA (red) and PVP (blue).

Although both coating strategies result in particle dispersions with moderate stability in the case of PVP and excellent stability for dispersions of nanorods coated with oleic acid and PMA, the latter one offers the advantage of free carboxy groups, which can be employed for a future conjugation of the antibody trastuzumab *via* EDC/sulfo-NHS linker chemistry, as discussed in the outlook section.

4.2.3 PlasMag Measurements

Phase lag spectra of nickel nanorod samples in EtOH coated with either oleic acid or lauric acid and without coating are compared in Fig. 40. According to the theoretical model presented by Schrittwieser ¹¹⁹, it can be seen, that the phase lag roughly follows an arctan dependence with increasing frequency of the external rotating magnetic field.

Due to the adhesion of molecules to the surface of the nickel nanorods, the hydrodynamic radius of the particles increases, thus resulting in increased phase lag, according to the theory. Since the coating carboxylic acids only produces a very thin layer (approximately 1-2 nm ^{120,121}) around the nanorods, no significant difference in the phase lag can be observed.

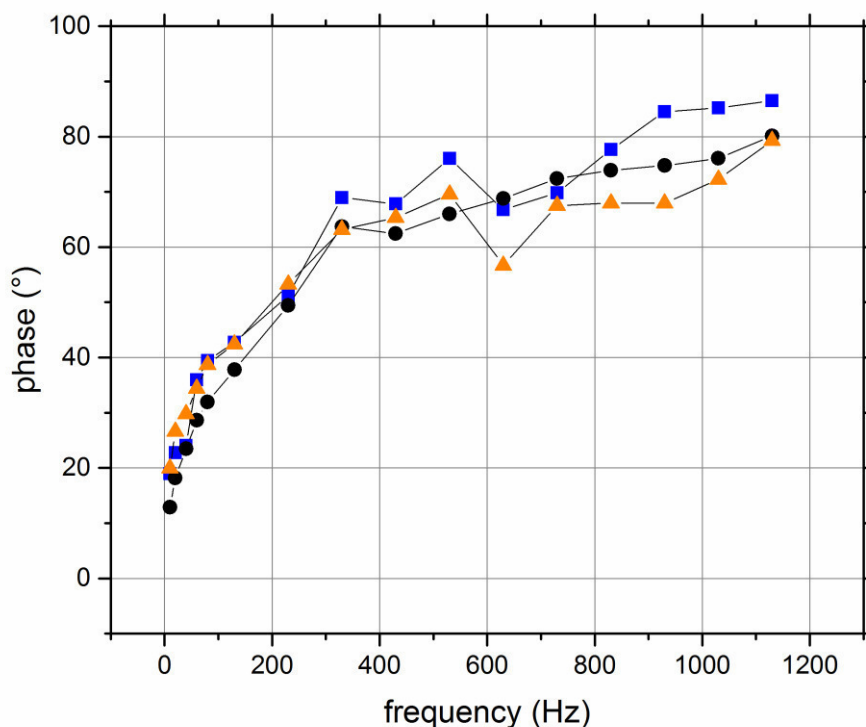


Figure 40 | Phase lag spectra of bare nanorods (▲) compared to nanorods coated with oleic acid (■) or lauric acid (●) in EtOH.

In Fig. 41, the phase lag spectra of PMA coated particles in Milli-Q water are compared. While the particles previously coated with fatty acids show the predicted arctan dependence (black and red indicators), the particles solely coated with PMA without prior fatty acid treatment clearly diverge from it (blue and green indicators). This finding suggests that the coating with fatty acids facilitates the subsequent surface modification with PMA, as it is assumed in the theory and depicted in Fig. 32. The smoother phase lag curve and the decreased slope at small frequencies indicate an increased particle dispersion stability. This, supports the results from the zeta potential measurements, as reported in the previous section.

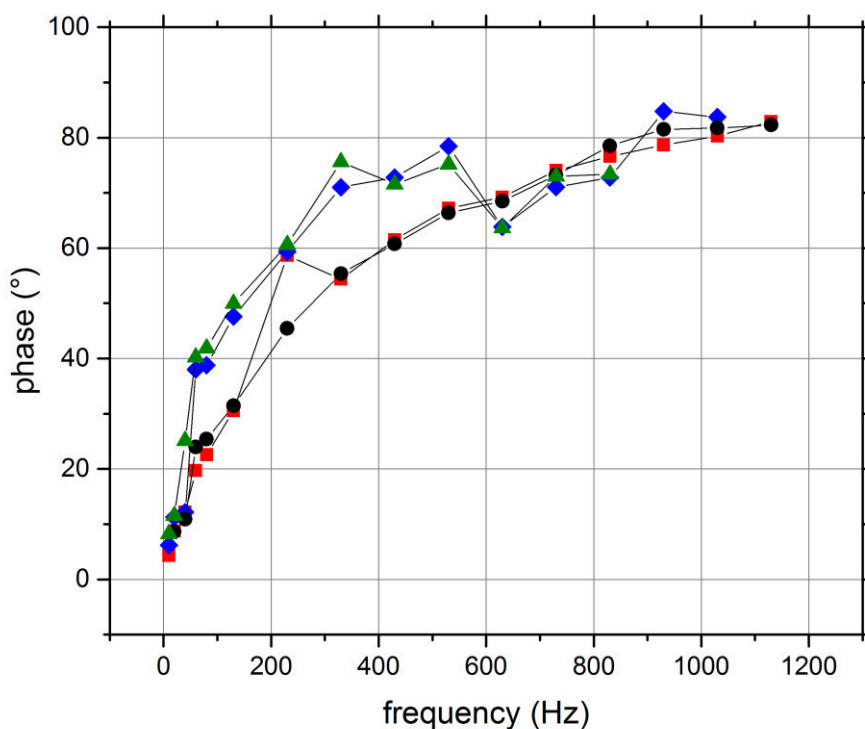


Figure 41 | Phase lag spectra of bare nanorods coated with modified PMA (1000 monomers per nm² nanoparticle surface, $\blacklozenge$), (5000 monomers per nm² nanoparticle surface, $\blacktriangle$) compared to nanorods coated with oleic acid and modified PMA ($\bullet$) or lauric acid and modified PMA ($\blacksquare$) in Milli-Q water.

The short-time stability of surface modified nanoparticle samples was analyzed by performing PlasMag measurements in regular intervals without previous redispersion. As can be seen in Fig. 42, colloidal dispersions of nanoparticles coated with oleic acid and PMA (1000 monomers/nm² of particle surface) remain stable for at least 24 hours at room temperature. The measurements were conducted in Milli-Q water. A stability over a period of 24 hours ensure a time span that is long enough for further surface modifications by antibodies.

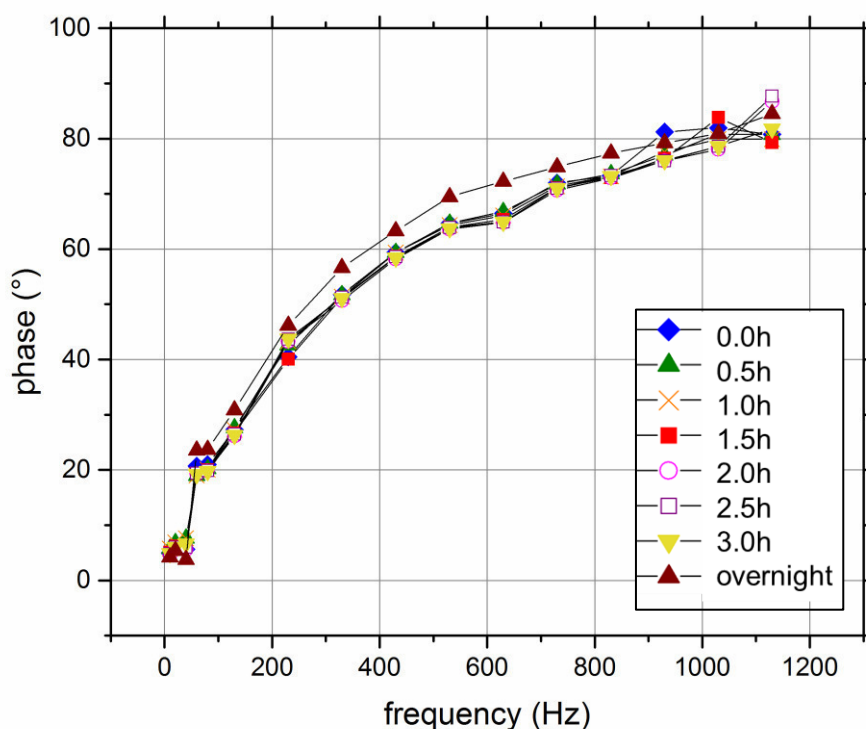


Figure 42 | Phase lag spectra of nickel nanorods coated with oleic acid and modified PMA (1000 monomers per nm² nanoparticle surface) over a period of 24 h in Milli-Q water.

The same result as described above was obtained for samples containing nanoparticles coated with lauric acid and PMA (1000 monomers/nm² of particle surface) in Milli-Q water, as can be seen in Fig. 43.

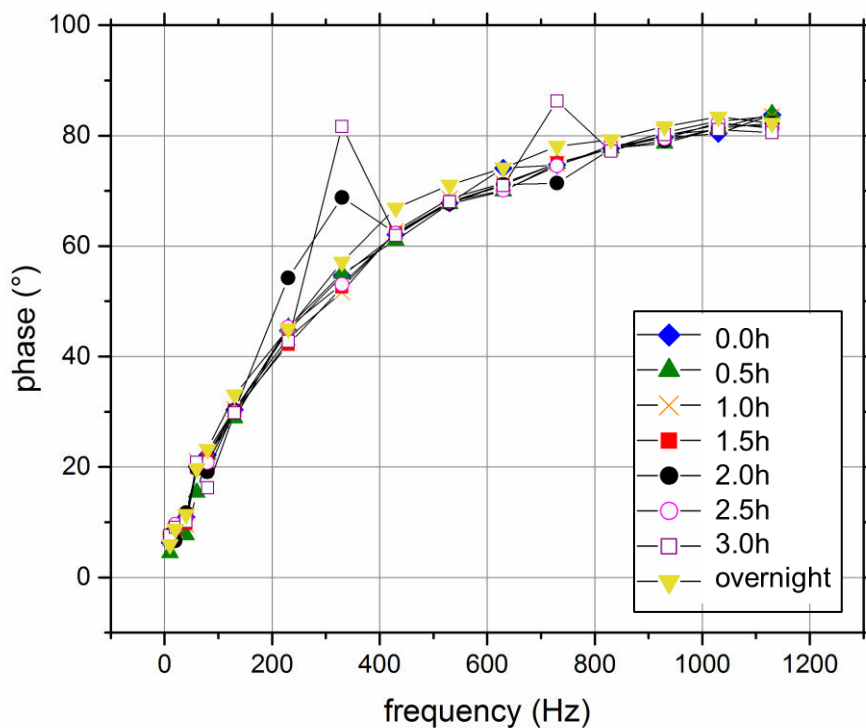


Figure 43 | Phase lag spectra of nickel nanorods coated with lauric acid and modified PMA (1000 monomers per nm² nanoparticle surface) over a period of 24 h in Milli-Q water.

Next to comparing the two employed fatty acid molecules, the number of applied PMA monomer units per nanorod surface unit can be varied. By this approach it can be examined if an increased amount of polymer comprising charged groups affects the nanorod dispersion stability. Here, it was observed that increasing the number of PMA monomers from 1000 to 5000 monomers per nm^2 of particle surface did not further increase the stability in colloidal dispersion, as can be seen in Fig. 44. The here measured phase lag is well comparable to the ones shown in the figures above for an employed monomer density of 1000 polymer monomers per nm^2 . The measurements were conducted in Milli-Q water.

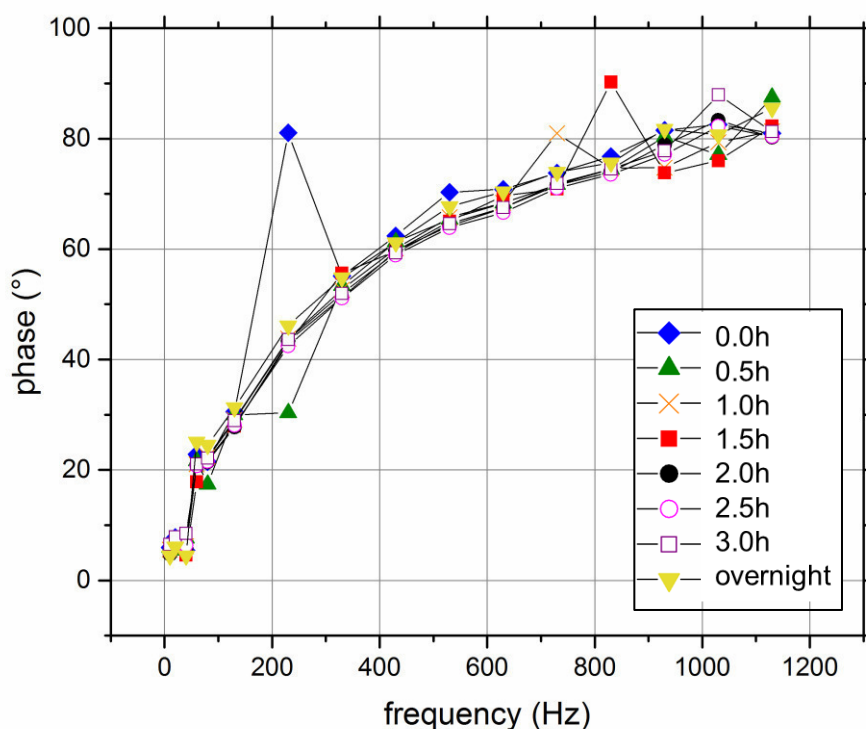


Figure 44 | Phase lag spectra of nickel nanorods coated with lauric acid and modified PMA (5000 monomers per nm^2 nanoparticle surface) over a period of 24 h in Milli-Q water.

Although particles solely coated with PMA without prior fatty acid treatment can be examined with the PlasMag measurement setup after a period of 24 hours, the obtained phase lag signal significantly diverges from the ideal arctan dependence, as depicted in Fig. 45 and Fig. 46. An increased phase lag is a result of an increased hydrodynamic nanorod size, which itself is a result of a favored agglomeration behavior. Furthermore, it can be seen that a phase lag value of about 70-80° is reached at a frequency of about 300 Hz and that this value does not further change for higher frequencies of the external rotating magnetic field. The measurements were conducted in Milli-Q water.

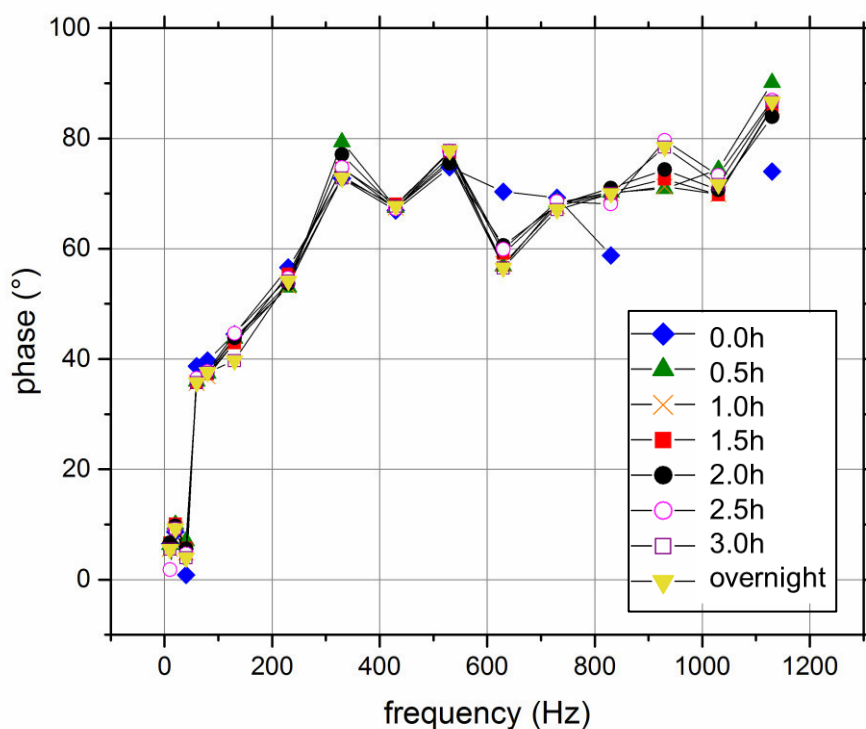


Figure 45 | Phase lag spectra of bare nickel nanorods coated with modified PMA (1000 monomers per nm² nanoparticle surface) over a period of 24 h in Milli-Q water.

Just as demonstrated in Fig. 45 and Fig. 46, an increase in the number of PMA monomers on the particle surface does not further stabilize colloidal dispersions of bare particles. Due to the low intensities of the signal, only the measurement at the start and at the end of the time interval are shown. Again, the measurements were conducted in Milli-Q water.

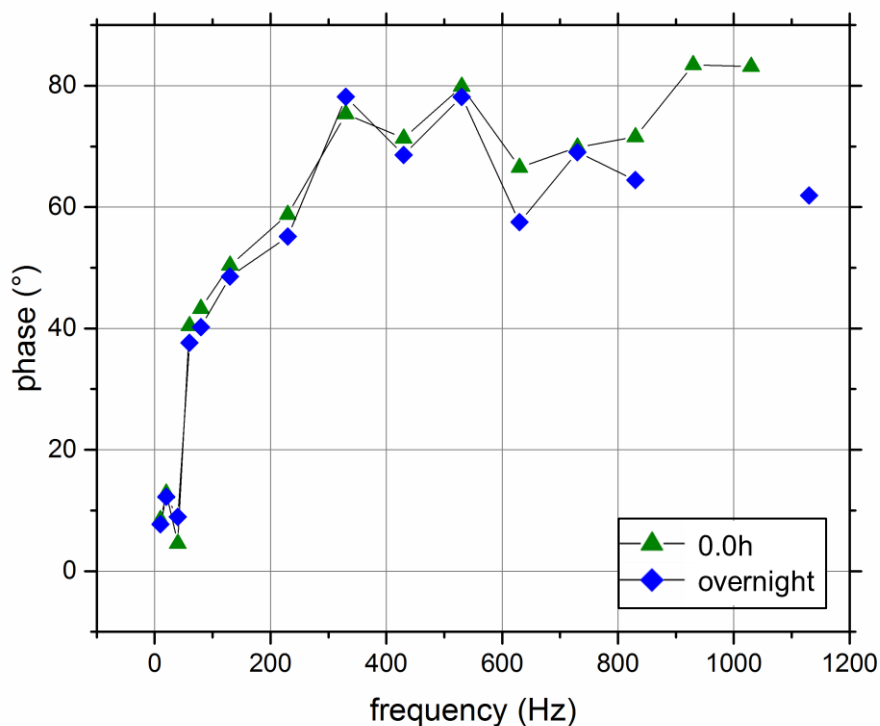


Figure 46 | Phase lag spectra of bare nickel nanorods coated with modified PMA (5000 monomers per nm² nanoparticle surface) over a period of 24 h in Milli-Q water.

5 Conclusion and Outlook

In this thesis, the electrochemical synthesis of nickel nanorods in a template based approach and their subsequent stabilization in aqueous colloidal dispersion is presented, which provides a basis for the functionalization with antibodies and ultimately leads to nanoprobes applicable for PlasMag measurements for biomarker POC testing.

Within the scope of this thesis, nickel nanoparticles were prepared in a three-step process by producing a nanosized porous aluminium oxide layer on an aluminium template *via* anodization. Through pulsed electrodeposition of nickel from a Nickel Watts bath, the pores in the template, which were created in the previous step, were filled and finally, the nanorods were released from the template. The size and shape of the nanorods was examined with electron microscopy and revealed particles with a length of approximately $201.5 \text{ nm} \pm 13.6 \text{ nm}$ and a diameter of approximately 15 nm. Then, the particles were stabilized in colloidal dispersion by modifying their surface chemistry.

For this purpose, the particles were coated with the water-soluble polymer poly(*N*-vinylpyrrolidone (PVP) or carboxylic acids like oleic acid and lauric acid and an amphiphilic polymer based on poly(isobutylene-*alt*-maleic anhydride (PMA). PVP adsorbs to the surface of nickel nanorods through its hydrophilic pyrrolidone group and not only prevents agglomeration of particles due to a layer of varying chain lengths, which cause steric repulsions, but also through a moderate degree of electrostatic repulsion between adjacent, similarly charged particles in the dispersion, as indicated by a zeta potential of 35 mV.

Carboxylic acids adsorb to the surface of nickel nanoparticles as carboxylates, where the two oxygen atoms are coordinated symmetrically to nickel atoms, forming covalent Ni-O bonds, as can be seen in Fig. 32. While the hydrophilic head groups adsorb to the nanoparticle surface, the hydrophobic tails form a hydrophobic layer around the particle, which is employed as a priming layer for the coating with modified PMA. The previous dodecylamine modification of the PMA polymer provides hydrophobic side chains in the PMA polymer, which are likely to align with those of the priming layer, due to hydrophobic van der Waals interactions, thereby creating the second layer, made of the PMA polymer. Hydrolyzed succinic anhydride groups within the PMA polymer yield negatively charged carboxy groups. A zeta potential of -88.64 mV was determined for oleic acid and PMA coated nanoparticles, which indicates a high stability of the aqueous nanoparticle dispersion due to electrosteric repulsions. Although the stabilization approach based on PVP yielded stable colloidal dispersions, the strategy relying on carboxylic acids and PMA is preferred, due to a higher reactivity of the free carboxy groups of the PMA polymer in contrast to the unreactive pyrrolidone groups of the PVP, which is needed for the subsequent conjugation of the nanorod surface by antibodies.

Preliminary antibody conjugation experiments were already conducted within the scope of this thesis (see appendix section 6.3 for experimental details). Here, the goal was to link

trastuzumab antibodies to the nanorod surface via EDC/sulfo-NHS linker chemistry as depicted in Fig. 47. Unfortunately, the PlasMag data obtained from preliminary conjugation experiments could not be evaluated. Here, an increased measured phase lag with respect to nanorods without antibody coating presents an indicator for antibody coating of the nanorod surface. Additionally, assay measurements to detect the corresponding antigen in solution will be needed to verify antibody linkage. Thus, a successful antibody linkage could not be confirmed experimentally. Most likely, this is due to a loss of particles during the dialysis steps, since a colorless solution was obtained instead of the originally clear, grey solution.

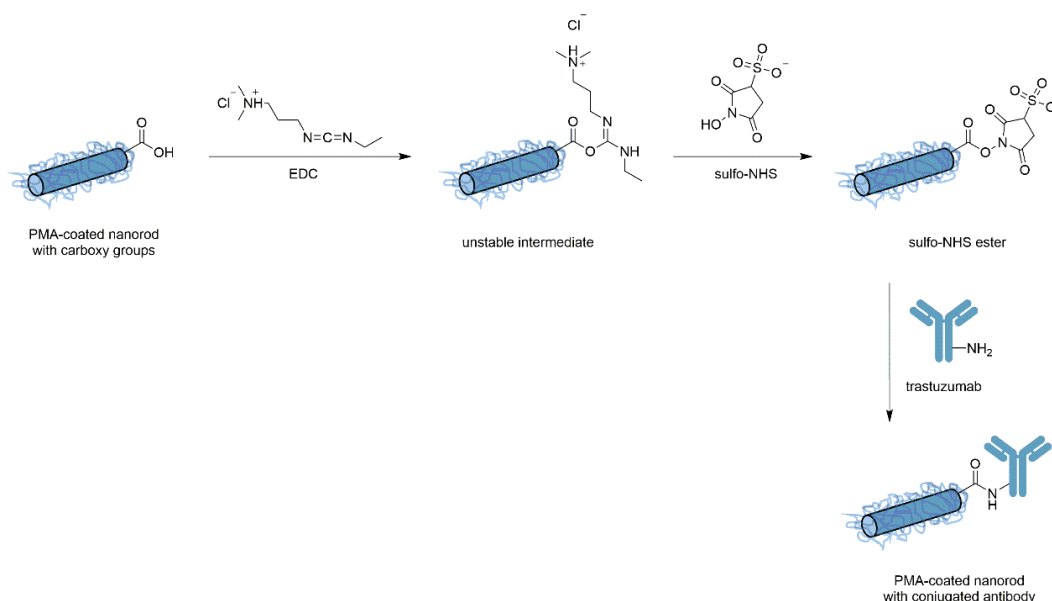


Figure 47 | Reaction scheme of the bioconjugation. EDC reacts with carboxylic acid groups on the nanoparticle to form an active *O*-acylisourea intermediate. The hydroxyl group on sulfo-NHS reacts with the active ester forming a sulfo-NHS ester intermediate, which undergoes a reaction with amine groups on the antibody to produce a stable amide linkage.

Thus, in a next step of synthesizing nanoparticles applicable for PlasMag measurements, the conjugation of the antibody must be optimized to yield a sufficient number of particles. For this reason, instead of dialyzing the particles, they could be magnetically separated and washed several times to minimize the loss, so that enough particles remain for the PlasMag measurements. By adding the antibody-nanoparticle conjugates to a sample spiked with the analyte sHER2 and comparing the phase lag spectra of unconjugated and conjugated particles, the outcome of the conjugation reaction can be determined. It is also possible to perform Fourier-transform infrared spectroscopy (FT-IR) or X-ray photoelectron spectroscopy (XPS) measurements to examine the nanorod surface.

Subsequently, it is also of importance to perform long-term stability measurements of the antibody-coated particles to gain insights on how long functionalized particles can be stored and then be employed for POC testing.

Not only carboxylic acids and similarly all kinds of fatty acids, but also alkylphosphonic acids were reported to bind to native nickel oxide surfaces ¹²². Since these molecules also comprise a phosphonic group in addition to the carboxy group, this additional functionality could be used for further surface modification approaches. Alternatively, polyacrylic acid (PAA) seems a promising candidate for nickel nanorod surface modification due to carboxyl groups in every monomer unit. The overgrowth of the nickel nanorod surface by silanization also presents a possibility for surface modification. Silane groups provided by (3-aminopropyl)triethoxysilane (APTES) and (3-aminopropyl) trimethoxysilane (APTMS), for example, form covalent bonds with hydroxyl groups present at the surface of the metal (oxide) template in a self-assembly process. Just like alkylphosphonic acids, an additional functionality is introduced and can be employed for further modifications.

Currently, the amount of chemicals employed for the coating procedures is based on calculations that assume the number of particles in the sample solution, since an instrument to conduct inductively coupled plasma mass spectrometry (ICP-MS) measurements was not available. ICP-MS would allow a determination of the nanoparticle concentration in the sample and would therefore lead to more precise instructions of the coating procedure and higher reproducibility if routinely employed.

To conclude, this thesis explains the idea and advantages of a POC testing device based on the rotational dynamics of magnetic nanoparticles functionalized with antibodies and describes the synthesis and stabilization of nickel nanorods in aqueous colloidal dispersion. Furthermore, this work summarizes the theoretical background of the template-based electrochemical synthesis of nickel nanorods and presents the different underlying principles for the stabilization of nanoparticles as well as functionalization strategies for the conjugation of antibodies. In addition, the theoretical basics of electron microscopy and the PlasMag measurement principle are briefly elucidated. Based on the presented measurements, the nanoparticle stabilization approaches based on the coating with the fatty acids oleic acid and lauric acid and PMA appear very promising for the subsequent antibody conjugation. By this method, suitable nanoprobe for the PlasMag measurement method can be obtained. Moreover, an outlook is given on how the coating process could be further optimized and variations of the surface chemistry for nanoparticle stabilization are also provided.

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List of Abbreviations

AC		alternating current
AIT		Austrian Institute of Technology
CE		counter electrode
DLVO		Derjaguin, Landau, Verwey, Overbeek
DNA		deoxyribonucleic acid
e.g.		exempli gratia
EGFR		Epidermal growth factor receptor
ELISA		Enzyme-linked Immunosorbent Assay
EU		European Union
h		hours
ICP-MS		Inductively Coupled Plasma Mass Spectrometry
IUPAC		International Union of Pure and Applied Chemistry
MWCO		Molecular Weight Cut Off
N _A		Avogadro constant
NP		nanoparticle(s)
PDI		Polydispersity index
POC		Point-of-care
RE		reference electrode
RMF		rotating magnetic field
ROMP		ring opening metathesis polymerization
SEM		Scanning Electron Microscope
SERS		Surface Enhanced Raman Scattering
WE		working electrode

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Appendix

Calculations for Surfactant coating

targeted surfactant molecules needed for one template
= variable · overall NR surface

mol of surfactant needed for one template
= $\frac{\text{targeted surfactant molecules needed for one template}}{N_A}$

g of surfactant needed for one template
= mol of surfactant needed for one template · molecular weight of surfactant

mg of surfactant needed for one template
= 1000 · g of surfactant needed for one template

ml of surfactant needed for one template
= $\frac{\text{g of surfactant needed for one template}}{\text{density of surfactant}}$

μl of surfactant needed for one template
= 1000 · ml of surfactant needed for one template

μl of surfactant needed for a quarter of one template
= $\frac{\mu\text{l of surfactant needed for one template}}{4}$

Calculations for PMA coating

Nickel nanorod characteristics:

$$\text{diameter (nm)} = 15$$

$$\text{length (nm)} = 200$$

$$\text{number of NR per template} = 1.52 \cdot 10^{12}$$

$$\text{surface per NR (nm}^2\text{)} = 2 \cdot r \cdot \pi \cdot l + 2 \cdot r^2 \cdot \pi$$

$$\text{overall NR surface (nm}^2\text{)} = \text{number of NR per template} \cdot \text{NR surface}$$

$$\text{overall NR stock solution volume per template} = \text{variable}$$

$$\text{NR per ml stock solution} = \frac{\text{number of NR per template}}{\text{overall NR stock solution volume per template}}$$

PMA polymer characteristics:

$$\text{molecular weight polymer (g/mol)} = 6000$$

$$\text{molecular weight monomer (g/mol)} = 154.16$$

$$\text{monomers per polymer molecule} = \frac{\text{molecular weight polymer}}{\text{molecular weight monomer}}$$

$$\text{PMA solution volume (ml)} = \text{variable}$$

$$\text{polymer weight in PMA solution (g)} = \text{variable}$$

$$\text{PMA solution mass concentration (g/ml)} = \frac{\text{polymer weight in PMA solution}}{\text{PMA solution volume}}$$

$$\begin{aligned} &\text{molar concentration polymer molecules (mol/l)} \\ &= \frac{\text{PMA solution mass concentration} \cdot 1000}{\text{molecular weight polymer}} \end{aligned}$$

$$\begin{aligned} &\text{molar concentration monomer molecules (mol/l)} \\ &= \text{monomers per polymer molecule} \cdot \text{Molar concentration polymer molecules} \end{aligned}$$

$$\text{number monomers per liter} = \text{molar concentration monomer molecules} \cdot N_A$$

$$\text{number monomers per ml} = \frac{\text{number monomers per liter}}{1000}$$

Calculator:

targeted monomers per nm^2 = variable

NR stock solution volume fraction (ml) = variable

NR number in stock volume fraction
= NR stock solution volume fraction $\cdot$ NR per ml stock solution

total NR surface (nm^2) in volume fraction
= surface per NR $\cdot$ NR number in stock volume fraction

total required monomer number
= targeted monomers per nm^2 $\cdot$ total NR surface (nm^2) in volume fraction

required fraction of PMA stock solution (ml) = $\frac{\text{total required monomer number}}{\text{number monomers per ml}}$

required fraction of PMA stock solution (μl)
= required fraction of PMA stock solution (ml) $\cdot$ 1000

PMA coating (1000 monomers per nm² nanoparticle surface) of oleic acid coated nanorods:

Ni nanorods	
diameter (nm)	15
radius (nm)	7,5
länge (nm)	200
surface per nanorod (nm ²)	9778,207134
number of nanorods per template	1,52E+12
overall nanorod surface (nm ²)	1,49E+16
overall nanorod stock solution volume per template (ml)	4
nanorods per ml stock solution	3,80E+11
PMA polymer	
g/mol für das gesamte Polymer	6000
g/mol für das Monomer	154,16
Monomere pro Polymermolekül	38,92060197
PMA solution volume (ml)	15
Polymer weight in PMA solution (g)	0,77
PMA solution mass concentration (g/ml)	0,051333333
PMA mass concentration (g/l)	51,33333333
Molar concentration polymer molecules (mol/l)	0,008555556
Molar concentration Monomers (mol/l)	0,332987372
Number monomers / l	2,01E+23
Number monomers / ml	2,01E+20
Calculator	
Targeted monomers per nm ²	1000
Nanorod stock solution volume fraction (ml)	0,1
Nanorod number in stock volume fraction	3,80E+10
Total nanorod surface (nm ²) in volume fraction	3,72E+14
Total required monomer number	3,71572E+17
Required fraction of PMA stock solution (ml)	0,00
Required fraction of PMA stock solution (µl)	1,85

PMA coating (1000 monomers per nm² nanoparticle surface) of lauric acid coated nanorods:

Ni nanorods	
diameter (nm)	15
radius (nm)	7,5
länge (nm)	200
surface per nanorod (nm ²)	9778,207134
number of nanorods per template	1,52E+12
overall nanorod surface (nm ²)	1,49E+16
overall nanorod stock solution volume per template (ml)	4
nanorods per ml stock solution	3,80E+11
PMA polymer	
g/mol für das gesamte Polymer	6000
g/mol für das Monomer	154,16
Monomere pro Polymermolekül	38,92060197
PMA solution volume (ml)	15
Polymer weight in PMA solution (g)	0,77
PMA solution mass concentration (g/ml)	0,051333333
PMA mass concentration (g/l)	51,33333333
Molar concentration polymer molecules (mol/l)	0,008555556
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Number monomers / l	2,01E+23
Number monomers / ml	2,01E+20
Calculator	
Targeted monomers per nm ²	1000
Nanorod stock solution volume fraction (ml)	0,1
Nanorod number in stock volume fraction	3,80E+10
Total nanorod surface (nm ²) in volume fraction	3,72E+14
Total required monomer number	3,71572E+17
Required fraction of PMA stock solution (ml)	0,00
Required fraction of PMA stock solution (µl)	1,85

PMA coating (5000 monomers per nm² nanoparticle surface) of lauric acid coated nanorods:

Ni nanorods	
diameter (nm)	15
radius (nm)	7,5
länge (nm)	200
surface per nanorod (nm ²)	9778,207134
number of nanorods per template	1,52E+12
overall nanorod surface (nm ²)	1,49E+16
overall nanorod stock solution volume per template (ml)	4
nanorods per ml stock solution	3,80E+11
PMA polymer	
g/mol für das gesamte Polymer	6000
g/mol für das Monomer	154,16
Monomere pro Polymermolekül	38,92060197
PMA solution volume (ml)	15
Polymer weight in PMA solution (g)	0,77
PMA solution mass concentration (g/ml)	0,051333333
PMA mass concentration (g/l)	51,33333333
Molar concentration polymer molecules (mol/l)	0,008555556
Molar concentration Monomers (mol/l)	0,332987372
Number monomers / l	2,01E+23
Number monomers / ml	2,01E+20
Calculator	
Targeted monomers per nm ²	5000
Nanorod stock solution volume fraction (ml)	0,1
Nanorod number in stock volume fraction	3,80E+10
Total nanorod surface (nm ²) in volume fraction	3,72E+14
Total required monomer number	1,85786E+18
Required fraction of PMA stock solution (ml)	0,01
Required fraction of PMA stock solution (µl)	9,26

PMA coating (1000 monomers per nm² nanoparticle surface) of bare nanorods:

Ni nanorods	
diameter (nm)	15
radius (nm)	7,5
länge (nm)	200
surface per nanorod (nm ²)	9778,207134
number of nanorods per template	1,52E+12
overall nanorod surface (nm ²)	1,49E+16
overall nanorod stock solution volume per template (ml)	4
nanorods per ml stock solution	3,80E+11
PMA polymer	
g/mol für das gesamte Polymer	6000
g/mol für das Monomer	154,16
Monomere pro Polymermolekül	38,92060197
PMA solution volume (ml)	15
Polymer weight in PMA solution (g)	0,77
PMA solution mass concentration (g/ml)	0,051333333
PMA mass concentration (g/l)	51,33333333
Molar concentration polymer molecules (mol/l)	0,008555556
Molar concentration Monomers (mol/l)	0,332987372
Number monomers / l	2,01E+23
Number monomers / ml	2,01E+20
Calculator	
Targeted monomers per nm ²	1000
Nanorod stock solution volume fraction (ml)	0,1
Nanorod number in stock volume fraction	3,80E+10
Total nanorod surface (nm ²) in volume fraction	3,72E+14
Total required monomer number	3,71572E+17
Required fraction of PMA stock solution (ml)	0,00
Required fraction of PMA stock solution (µl)	1,85

PMA coating (5000 monomers per nm² nanoparticle surface) of bare nanorods:

Ni nanorods	
diameter (nm)	15
radius (nm)	7,5
länge (nm)	200
surface per nanorod (nm ²)	9778,207134
number of nanorods per template	1,52E+12
overall nanorod surface (nm ²)	1,49E+16
overall nanorod stock solution volume per template (ml)	4
nanorods per ml stock solution	3,80E+11
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g/mol für das gesamte Polymer	6000
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Total nanorod surface (nm ²) in volume fraction	3,72E+14
Total required monomer number	1,85786E+18
Required fraction of PMA stock solution (ml)	0,01
Required fraction of PMA stock solution (µl)	9,26

Preliminary Experiments for the Conjugation of the Antibody

Calculations for the Conjugation of the Antibody

$$\text{number of NR per template} = 1.52 \cdot 10^{12}$$

$$\text{fraction of template employed} = \text{variable}$$

$$\text{used sample volume (ml)} = \text{variable}$$

$$\text{particles in sample volume} = \frac{\text{number of NR per template}}{\text{fraction of template employed}}$$

$$\text{trastuzumab concentration (mg/ml)} = 20$$

$$\text{trastuzumab volume (ml)} = \frac{\text{needed trastuzumab}}{\text{trastuzumab concentration}}$$

$$\text{EDC molecules per particle} = 10000$$

$$\text{trastuzumab molecules per particle} = 200$$

$$\text{sulfo - NHS molecules per particle} = 30000$$

$$\text{needed EDC molecules} = \text{EDC molecules per particle} \cdot \text{particles in sample volume}$$

$$\begin{aligned} &\text{needed trastuzumab molecules} \\ &= \text{trastuzumab molecules per particle} \cdot \text{particles in sample volume} \end{aligned}$$

$$\begin{aligned} &\text{needed sulfo – NHS molecules} \\ &= \text{sulfo – NHS molecules per particle} \cdot \text{particles in sample volume} \end{aligned}$$

$$\begin{aligned} &\text{mass of linker molecules (g)} \\ &= \frac{\text{needed linker molecules}}{N_A} \cdot \text{molecular weight of linker molecule (g/mol)} \end{aligned}$$

$$\text{mass of linker molecule (mg)} = 1000 \cdot \text{mass of linker molecules (g)}$$

$$\text{mol of linker molecule (mol)} = \frac{\text{mass of linker molecules (g)}}{\text{molecular weight of linker molecule (g/mol)}}$$

Surface Functionalization of Nickel Nanorods

For the covalent conjugation of the monoclonal trastuzumab antibodies, the carboxy groups of the PMA backbone were activated with EDC/sulfo-NHS to react with the amine groups present on the antibody resulting in the formation of an amide bond. The bioconjugation reaction was carried out in a MES buffer solution (50 mM) at pH 5.5.

To 1 ml PMA-coated nanoparticle solution in Milli-Q water, EDC and sulfo-NHS were added and the mixture was incubated (15 min, room temperature). Then, the solution was transferred into a dialysis membrane (MWCO 1,000,000 Da) and was dialyzed against 500 ml MES buffer for 30 min. The solution was transferred back into a glass vial and the desired volume of trastuzumab (20 mg/ml) with 200 μ l MES buffer (pH 5.5) were added to the mixture and incubated (100 min, room temperature; then overnight, 4 °C)). Afterwards, the solution was transferred into a dialysis membrane (MWCO 1,000,000 Da) and was dialyzed against 500 ml MES buffer for overnight.