



MASTERARBEIT | MASTER'S THESIS

Titel | Title

Systematische Untersuchung molekular geprägter Polymer-Nanopartikeln (nano-MIPs) für die Detektion von Insulin

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree
programme code as it appears on the
student record sheet:

UA 066 863

Studienrichtung lt. Studienblatt | Degree
programme as it appears on the student
record sheet:

Masterstudium Biologische Chemie

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Acknowledgement

I would like to take the opportunity here to express my gratitude to those without whom this work would not have been possible:

First and foremost, sincere thanks go to Univ.-Prof. Dr. Peter Lieberzeit, who gave me the wonderful opportunity to work in his group, and I am incredibly grateful to him for being an inspiring and attentive leader to me. I was very happy to work under his guidance and very glad that fate brought me to this laboratory.

I am especially thankful to Julia Völkle for her high level of professionalism, clear and thoughtful approach to work, as well as for teaching me a number of laboratory techniques that were new to me. Her support and involvement in this project inspired me, and her approach to work became an example of the kind of specialist I would like to become.

Furthermore, I would like to thank the entire Lieberzeit working group for the warm, friendly, and very comfortable scientific atmosphere.

In addition, I would like to thank the Institute of Materials Chemistry & Research for providing access to instrumentation used for several necessary measurements.

Finally, heartfelt thanks to my family and friends for their support and presence throughout my entire academic journey.

List of Abbreviations

ACN	Acetonitrile
APM	Acrylamide monomer
APS	Ammonium persulfate
APTES	3-Aminopropyltriethoxysilane
BIS	N,N'-Methylenebis(acrylamide)
BSA	Bovine serum albumin
CPA	4-cyano-4-(phenylcarbonothioylthio)pentanoic acid
DLS	Dynamic light scattering
DMF	Dimethylformamide
EtOH	Ethanol
FRP	Free radical polymerization
GA	Glutaraldehyde
HA	High-affinity fraction
INS	Insulin
LA	Low-affinity fraction
MIP	Molecularly Imprinted Polymer
MQ	Milli-Q water
NIPAm	N-Isopropylacrylamide
PBS	Phosphate-buffered saline
PDI	Polydispersity index
QCM	Quartz crystal microbalance
RAFT	Reversible addition-fragmentation chain-transfer
RT	Room temperature
SEM	Scanning electron microscopy
SG	Silica gel
SMA	SubMiniature version A
SPE	Solid-phase extraction
TBA _m	N-tert-Butylacrylamide
TEMED	Tetramethylethylenediamine
UV	Ultraviolet
ζ- potential	Zeta potential

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1. Introduction

1.1 Background and Motivation

1.1.1 Molecular recognition as a universal principle in biology and analytical chemistry

Molecular recognition is a fundamental process that underlies all biology. Virtually every interaction in living systems from enzyme function to immune response is based on the ability of certain molecules to selectively recognize others. This selectivity ensures the specificity of biochemical reactions and the stability of physiological processes. In essence, molecular recognition is a universal principle, without which the coordinated functioning of cells and entire organisms would be impossible.¹

Classical examples of such interactions include enzyme–substrate binding, hormone–receptor interactions, as well as the formation of protein–protein and protein–DNA complexes. A particularly striking example is the behavior of antibodies, which can distinguish even the smallest structural differences between molecules. Despite the diversity of functions and structures, all these systems share a common principle: complementarity in shape, charge, and the spatial distribution of chemical groups.²

Molecular recognition is made possible by the combination of several factors: geometric compatibility, electrostatic charge distribution, hydrogen bonding, and hydrophobic interactions.³ These parameters together create the high specificity required to distinguish even structurally similar compounds. This behavior is characteristic not only of proteins, but also of nucleic acids, lipids, carbohydrates, and small molecules involved in regulatory cascades.

In biochemical systems, recognition is often accompanied by conformational changes or activation of functional domains. This makes the process not just passive recognition, but an active element of regulation. Through this mechanism, the organism can precisely distinguish between "self" and "non-self", regulate metabolism, and maintain homeostasis.⁴

Understanding the principles of molecular recognition has paved the way for the development of artificial systems capable of mimicking this process. This is particularly relevant in analytics and sensing, where reliable and selective detection of specific molecules in complex mixtures is essential. Since nature has mastered the art of precise and efficient molecular recognition, it is logical to strive to transfer this principle into synthetic materials.

One approach to implementing this idea is molecular imprinting, a method that enables the creation of synthetic structures capable of recognizing specific compounds. This technology has become the foundation for a new generation of chemical and biosensors aimed at the selective detection of biomolecules.⁵

1.1.2 Molecular imprinting as a synthetic alternative

The development of robust and selective molecular recognition materials has become one of the key challenges in analytical chemistry and sensor technologies. Classical biomolecules such as antibodies exhibit high specificity, but they are inherently unstable outside biological environments, sensitive to temperature and pH, and require stringent storage conditions. These limitations restrict their use, especially in devices designed for long-term operation or deployment in harsh environments.⁶

In response to these constraints, an approach known as molecular imprinting was developed. It enables the fabrication of polymeric materials with a "memory" for target molecules by forming specific binding sites during the polymerization process. These sites exhibit spatial and chemical complementarity to the template molecule and are capable of repeatedly performing selective binding after the template is removed.⁷ The imprinting method using the solid-phase approach is described in Section 1.2.1.

This principle is comparable to creating a synthetic analogue of antibodies but based on polymers. Such materials are significantly more stable, do not require refrigeration, are cheaper to produce, and can be adapted to a wide variety of application conditions.⁸

Today, molecular imprinting is applied in the development of sensors, controlled drug release systems, water purification⁹, and in analytical chemistry for the separation and detection of compounds in complex matrices.¹⁰

The method of molecular imprinting began to develop actively in the 1970s and 1980s,¹ when the first fundamental studies on synthetic receptors based on polymers were published. The most frequently cited works in this context are those of the Wulff¹¹ and Mosbach¹², groups, who independently demonstrated the feasibility of stabilizing a specific monomer arrangement around a template molecule.

The first imprinted polymers were synthesized using bulk polymerization, where the entire reaction mixture is polymerized into a monolith that is subsequently ground and washed. Despite its simplicity, this approach has several limitations: the resulting particles exhibit high polydispersity, and the active binding sites are often buried within the material, limiting their accessibility to target molecules.¹³ Later, alternative methods were developed, including precipitation polymerization, which forms more uniform microspheres and solid-phase synthesis,¹⁴ which enables immobilization of the template on a solid support and results in more selective and homogeneous structures.

In the context of this study, special emphasis is placed on solid-phase synthesis, as it enables the production of nanoparticles with a high degree of size control and with binding sites exposed on the

surface. This is particularly important when working with large and sensitive targets such as hormones or proteins.¹⁵

The choice of polymerization strategy directly influences the properties of the final material: binding kinetics, selectivity, stability, and suitability for integration into sensor platforms. Classical methods based on free-radical polymerization (FRP) are still widely used, but they are inferior in controllability and reproducibility to more advanced techniques such as Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerization.¹⁶

1.1.3 Insulin as a complex target

Insulin is one of the key hormones regulating metabolism in humans and animals. It is a protein molecule composed of two polypeptide chains (A and B), linked by disulfide bridges (**Figure 1.1**). Its biological activity and ability to bind to receptors directly depend on its spatial conformation and the preservation of its tertiary structure.



Figure 1. 1: Three-dimensional structure of human insulin (PDB ID: 3I40). The A-chain (21 amino acids) is shown in blue, the B-chain (30 amino acids) in orange. Disulfide bridges are indicated by yellow sulfur atoms and grey connecting lines.

Precisely because of this structural sensitivity, insulin is considered a challenging target for the development of molecular recognition systems. Changes in pH, temperature,¹⁷ or exposure to solvents¹⁸ can lead to denaturation of its structure, making reliable recognition impossible in unstable environments.

For molecular imprinting-based systems, this presents a serious problem: the template must retain its structure throughout all stages of synthesis so that the imprint in the polymer corresponds to the native (biologically active) form of the molecule. However, most imprinting methods were originally developed for small molecules, which are more stable and less prone to structural deformation.¹⁹

Insulin, as a medium-sized protein (~5.8 kDa), contains both hydrophobic and charged regions, which complicates the formation of stable complexes with monomers in solution. In addition, due to its flexible nature and tendency to aggregate, it is crucial to avoid conditions that promote dimer or

hexamer formation.²⁰ Despite these challenges, the development of polymer systems capable of selectively recognizing insulin remains a highly relevant goal, especially in the context of creating sensors for real-time glucose and insulin monitoring. Such systems may form the basis of non-invasive diagnostic devices, including wearable sensors.²¹

1.1.4 What are nano-MIPs and why are they superior

Recent advances in molecular imprinting have enabled the creation of materials that combine the selectivity of antibodies with the robustness and scalability of synthetic systems. A particularly important development was the emergence of nanostructured MIP particles, known as nano-MIPs - Molecularly Imprinted Polymer nanoparticles that offer several advantages over traditional, bulk MIP structures.²²

Nano-MIPs typically range in size from a few tens to several hundred nanometers (for example, particles with diameters of 28–37 nm²³, 120–150 nm²⁴, and even up to 350 nm²⁵ have been reported) and can be synthesized with a high degree of control over shape, size, and the distribution of active sites. Due to their small size, nano-MIPs possess a significantly higher specific surface area (i.e., surface area per unit mass or volume) compared to bulk structures. This means that, for an equal amount of material, more binding sites are accessible on the surface of the particles. As a result, they enable faster mass transfer, improved binding kinetics, and increased sensitivity — all of which are very important when working with large and delicate targets such as proteins and peptides.

One of the key factors determining the selectivity of nano-MIPs is that their binding sites are predominantly formed on the particle surface. This reduces the risk of target molecules becoming "trapped" inside the polymer, as is often the case in bulk polymerization. Consequently, nano-MIPs offer faster response times and more efficient recognition in sensor applications.²⁶

In addition, nano-MIPs exhibit high stability in a variety of environments: they can be designed to function in organic solvents, and non-physiological temperatures and pH. This makes them particularly attractive for the development of wearable sensors, bioanalyzers, and integrated systems for continuous biomarker monitoring.²⁷

The production of nano-MIPs is typically carried out using a solid-phase synthesis approach (see Section 1.2), in which the template is immobilized on a solid support, and only those particles that correctly form binding sites are selectively eluted from the reaction mixture. This significantly improves the homogeneity and reproducibility of the synthesized material and eliminates the need for complex multi-step purification.¹⁴

1.1.5 Motivation for this study

Modern trends in analytical chemistry and biosensing demand the development of stable, selective, and scalable recognition materials capable of functioning under physiological and non-physiological conditions. In this context, MIPs are considered a promising alternative to bioreceptors, especially for real-time sensor applications.

One of the most interesting yet challenging targets for molecular imprinting is insulin.²⁸ Its relatively small molecular weight, high sensitivity to environmental conditions, and tendency to denature and aggregate make it particularly difficult to create stable and reproducible binding sites. At the same time, selective detection of insulin is of great importance in medicine, especially in the context of monitoring glucose–insulin regulation.

To overcome these limitations, the present study implemented the synthesis of nano-MIPs using a solid-phase approach and RAFT polymerization, aiming to produce stable, colloiddally dispersed particles with high reproducibility. These nanoparticles exhibit enhanced selectivity due to the formation of binding sites predominantly on their surface.

A central role in this work is played by the Quartz Crystal Microbalance (QCM) technique, which enables real-time monitoring of insulin binding without the need for labeling.²⁹ In addition to the traditional approach involving surface-immobilized insulin, this study also applies a competitive assay, in which the interaction between the target and the MIP occurs in solution.²⁴ This method allows for a more accurate assessment of the accessibility of binding sites on the nanoparticles and their affinity for the target molecule.

When the insulin concentration is varied at a fixed nano-MIP concentration, a change in signal is observed, reflecting the level of competition between free and immobilized insulin. This enables not only the evaluation of binding affinity but also the analysis of polymer site saturation.

1.2 MIPs: principles, properties, and synthesis methods

1.2.1 Fundamental principle of molecular imprinting

MIPs are synthetic materials capable of selectively recognizing target molecules due to the presence of binding sites within their structure that are complementary to the target in both shape and chemical functionality. These sites are formed during the polymerization process in the presence of a template molecule.³⁰

To visually illustrate the principle of imprinting, a simplified schematic representation is shown in **Figure 1.2**,³¹ outlining the key stages of MIP formation: from the pre-polymerization complex of monomers and template to polymer formation and subsequent target rebinding.

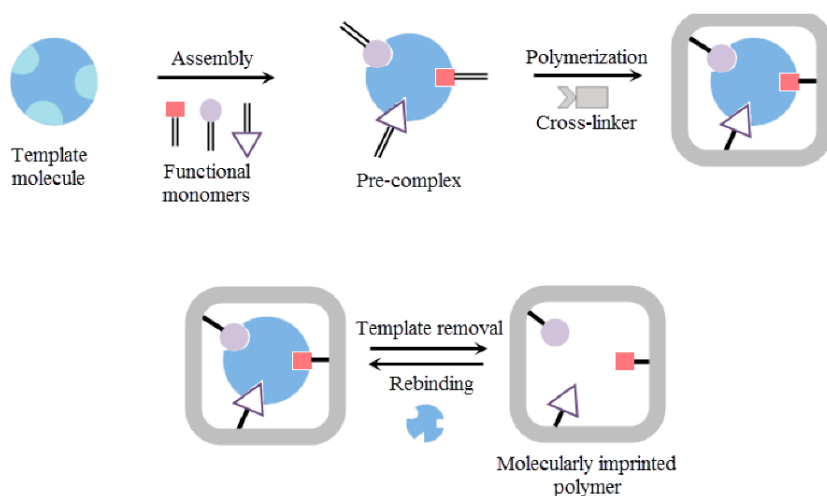


Figure 1. 2: Schematic representation of the molecular imprinting process: pre-complex formation, polymerization, template removal, and selective target rebinding.³¹

Upon addition of crosslinkers and initiators to the reaction mixture, polymerization is initiated, "locking" the monomers in their specific arrangements, thereby creating cavities capable of recognizing the original molecule.

Template removal is typically carried out by extraction with organic solvents (for small-molecule templates) or, in the case of proteins, via stepwise elution based on affinity differences for example, using a pH gradient from 5 to 8.³² These gradient-based strategies, including pH or temperature steps, are particularly relevant for solid-phase MIP synthesis, where low-affinity particles are released under mild conditions (e.g., neutral pH or room temperature), while high-affinity fractions require elevated pH or temperature (~65 °C) to be eluted.³³

One of the most successful solutions for such systems is solid-phase molecular imprinting, in which the template molecule is immobilized on the surface of a solid substrate. This ensures uniform orientation and facilitates the subsequent separation of the synthesized particles. **Figure 1.3** presents three strategies of covalent target immobilization, adapted from the work of Canfarotta et al., involving

amino, carboxyl (via carbodiimide coupling using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS)), or thiol functional groups (e.g., succinimidyl iodoacetate). In this study, the glutaraldehyde-based route (via amino groups) corresponding to the first strategy was applied. Additionally, unlike the original method, silica gel was used as the solid substrate instead of glass beads.

A key advantage of molecular imprinting lies in the possibility of designing polymers specific even to challenging targets such as peptides, hormones, and proteins. However, successful implementation requires consideration of multiple factors: target size and flexibility, charge, hydrophilicity, and sensitivity to pH and temperature. The selection of monomers and polymerization conditions must be precisely tailored.¹⁹ In this work, functional monomers and synthesis parameters were selected to enable selective interaction with insulin while maintaining the stability and uniformity of the polymer particles. A detailed description of the methodology is provided in the subsequent sections.

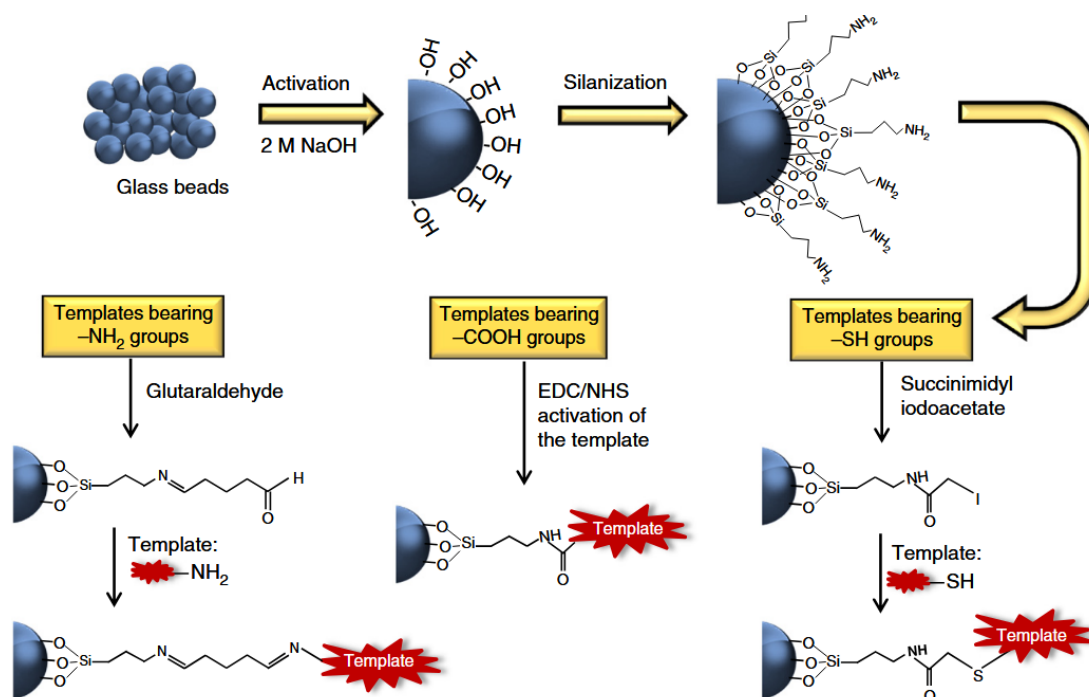


Figure 1. 3: Possible strategies for covalent immobilization of the template on a solid support: via amino groups (glutaraldehyde), carboxyl groups (EDC/NHS), or thiol groups (succinimidyl iodoacetate).¹⁹

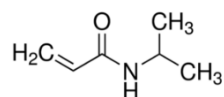
1.2.2 Structure and properties of nano-MIPs

The successful formation of highly selective nano-MIPs depends not only on the choice of target molecule, but also on the careful selection of monomers, synthesis conditions, and post-processing parameters. Since the binding sites are embedded in a rigid three-dimensional polymer matrix, the architecture of this matrix directly influences key properties such as selectivity, stability, hydrodynamic size, and suitability for analytical applications.

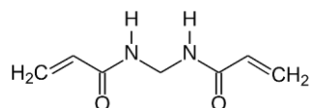
In this work, the synthesis scheme was based on RAFT polymerization in dimethylformamide (DMF), employing the iniferter concept. The RAFT agent used was 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPA), which simultaneously served as an initiator, chain transfer agent, and terminator, enabling narrow molecular weight distribution and controlled polymer structure.³⁴

The monomer mixture included the following components:

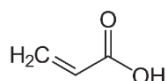
- N-isopropylacrylamide (NIPAm) — the main backbone monomer, imparting thermoresponsive properties;³⁵



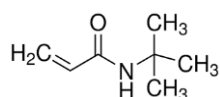
- N,N'-methylene-bisacrylamide (BIS) — a crosslinker forming the three-dimensional polymer network;³⁶



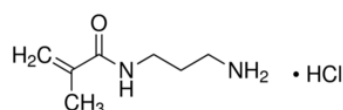
- Acrylic acid (AA) — provides ionic interactions;³⁵



- N-tert-butylacrylamide (TBAAm) — enhances hydrophobic interactions;³⁷



- N-(3-aminopropyl)-methacrylamide hydrochloride (APM) — contributes to hydrogen bonding and assists in monomer orientation.³⁸



1.3 RAFT polymerization for MIP synthesis

RAFT polymerization is a method of controlled radical polymerization that allows for precise regulation of polymer chain growth. Unlike conventional free-radical polymerization, where chains grow in a random and uncontrolled manner, RAFT enables synchronous and uniform formation of macromolecules.³⁹ This is particularly important for the synthesis of molecularly imprinted polymers in nanoscale format (nano-MIPs), where particle uniformity and reproducibility are critical parameters.

1.3.1 Mechanism of RAFT polymerization

The RAFT process is based on the use of a RAFT agent — a compound with the general structure $ZC(=S)S-R$, which participates in reversible reactions with growing polymer chains.⁴⁰ In this work, CPA (4-cyano-4-(phenylcarbonothioylthio)pentanoic acid) was used as a photo-iniferter. As a result, no external initiator was required, since polymerization was initiated under UV irradiation (312 nm), which induces homolytic bond cleavage and the formation of a primary radical.

The core mechanism of RAFT polymerization is illustrated in **Figure 1.4**:⁴¹

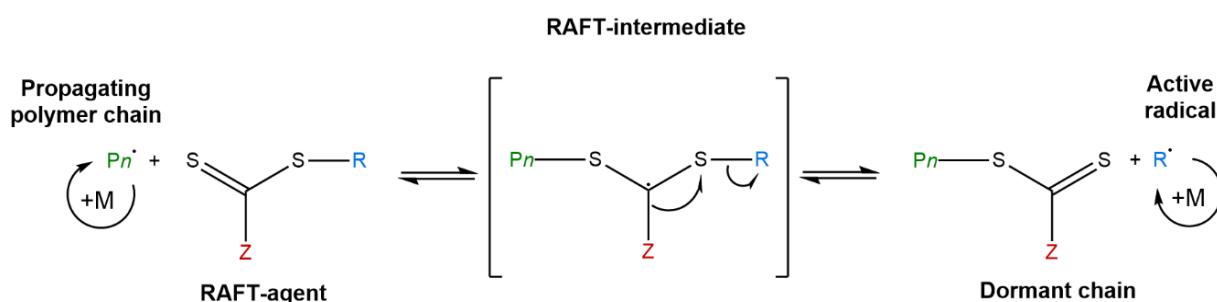


Figure 1. 4: Schematic representation of the RAFT mechanism: a radical reacts with a RAFT agent containing a thiocarbonylthio group, forming an intermediate adduct and releasing a new radical capable of initiating the growth of a new chain (Z — stabilizing group, R — homolytical leaving group, M — monomer).⁴²

The RAFT process involves several reversible steps:⁴²

1. Initiation: The RAFT agent spontaneously forms an active radical that initiates the growth of the first chain.
2. Addition to the RAFT agent: The growing radical chain attacks the C=S bond of the RAFT agent, forming an unstable radical intermediate.
3. Fragmentation: Homolytic cleavage of the C–S bond occurs, releasing an R• radical capable of initiating a new chain. The original chain at this point becomes temporarily inactive a so-called dormant species.
4. Dynamic equilibrium: Throughout the reaction, active and dormant chains coexist in solution, continuously interconverting. This equilibrium is the key to control: at any given moment, only a small fraction of chains is active, reducing the likelihood of side reactions and ensuring uniform polymer growth.

1.3.2 Chemical nature of the RAFT agent

Sulfur-containing compounds used as RAFT agents have the ability to stabilize radical intermediates through delocalization of electron density across sulfur atoms.⁴³ This feature enables reversible radical transfer, which is essential for the controlled growth of polymer chains.

For clarity, the structure of the RAFT agent CPA is shown in **Figure 1.5**, with the Z-group highlighted in red and the R-group in blue.

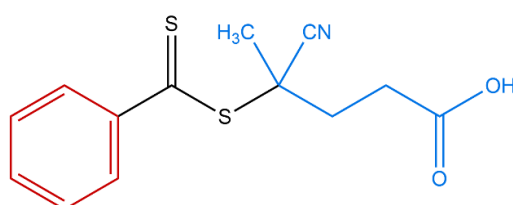


Figure 1. 5: Structure of the RAFT agent CPA.

The two key structural elements of a RAFT agent are the Z and R groups, each playing a distinct role in the reaction mechanism:

- The Z-group influences the reactivity of the agent and stabilizes intermediate species. Its selection depends on the type of monomers used. In this study, the RAFT agent CPA was used, in which the Z-group is a phenyl ring. This structure classifies CPA as a trithiocarbonate, which has been shown to be highly effective for the polymerization of methacrylamide-based monomers, including NIPAm and TBA_m.⁴²
- The R-group, on the other hand, must act as a good leaving group and be capable of initiating new chain growth. In CPA, this role is fulfilled by an α-cyano-substituted fragment, which facilitates efficient polymerization initiation.⁴⁴

1.3.3 Advantages of RAFT and justification for method selection

Unlike conventional free-radical polymerization (FRP), the RAFT method enables architectural control and high reproducibility even when working with sensitive or functional monomers. FRP-based approaches are characterized by uncontrolled chain growth, high polydispersity, and limited compatibility with hydrophilic or charged monomers.^{44, 45} These limitations can be critical in the synthesis of molecularly imprinted polymers, where the spatial organization of the polymer matrix directly affects molecular recognition selectivity.

In contrast, RAFT polymerization ensures uniform macromolecular growth, temporal control of active chains, and compatibility with organic solvents. This allows for the successful incorporation of various monomers such as amides, carboxylic acids, amines, and hydrophobic components while maintaining stable particle morphology and functional performance.⁴⁶ The monomer composition, its chemical characteristics, and their influence on particle properties were discussed in Section 1.2.2.

1.4 QCM for analyzing nano-MIP binding to insulin

The Quartz Crystal Microbalance (QCM) is a highly sensitive analytical technique based on the piezoelectric effect of quartz. This effect involves the generation of electric potential upon mechanical deformation of a quartz crystal, and conversely, oscillation of the crystal at a specific resonant frequency upon application of an alternating voltage. When molecules adsorb onto the crystal surface, the added mass causes a shift in this frequency. Thus, the QCM acts as a signal transducer, while the functionalized surface serves as the sensitive element.⁴⁷

Thanks to its miniaturization potential, nanogram-level sensitivity, and real-time measurement capability, the QCM has become a widely used method in biosensing, particularly for molecular recognition tasks including the analysis of proteins, hormones, and other biomarkers.⁴⁸

When an analyte specifically binds to the functionalized surface, the event is registered as a measurable frequency shift. The general principle is shown in **Figure 1.6** (adapted from Akgönüllü et al., 2022).⁴⁹

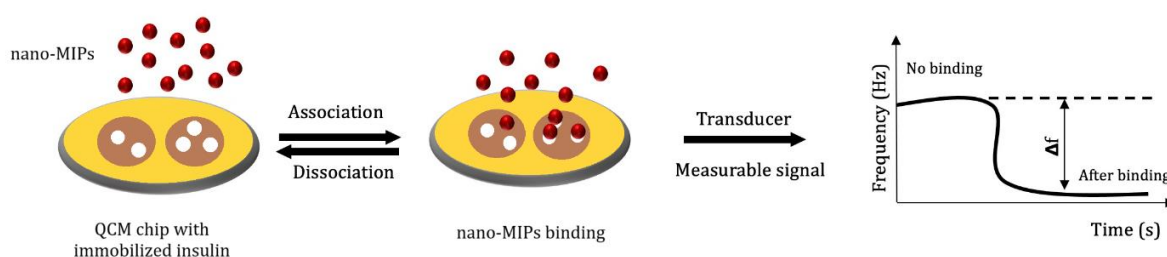


Figure 1.6: Working principle of the QCM sensor: binding of target molecules leads to a decrease in resonant frequency (Δf), which is recorded as the analytical signal.⁴⁹

The physical basis of this technique is described by the Sauerbrey equation⁵⁰, which quantitatively relates the change in frequency to the change in surface mass:

$$\Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\mu\rho}} \quad (1.4.1)$$

where:

Δf — frequency shift (Hz)

f_0 — fundamental resonant frequency of the crystal (Hz)

Δm — mass change on the surface (g)

A — active area between the electrodes (cm^2)

μ — shear modulus of quartz ($\mu = 2.947 \times 10^{11} \text{ g} \cdot \text{cm}^{-1} \cdot \text{s}^{-2}$)

ρ — density of quartz ($\rho = 2.648 \text{ g} \cdot \text{cm}^{-3}$).

In practice, this equation is often simplified as:

$$\Delta m = -C \frac{\Delta f}{n} \quad (1.4.2)$$

where:

C — sensitivity constant (depends on crystal properties)

n — overtone number

It should be noted that equation (1.4.1) is only valid under specific conditions: the deposited layer must be thin, rigid, and non-viscous. When dealing with biomolecules or nanoparticles, these assumptions are often violated. In particular, when a viscous or soft layer forms on the surface, nonlinear deviations from Sauerbrey behavior are observed.

To describe such cases, the Kanazawa–Gordon model^[51], is used, which accounts for the viscosity and density of the liquid, as well as acoustic wave damping in the surrounding medium:

$$\Delta f = -f_0^{3/2} \sqrt{\frac{\eta_L \rho_L}{\pi \mu \rho}} \quad (1.4.3)$$

where:

η_L — viscosity of the liquid (N·s·m⁻²)

ρ_L — density of the liquid (kg·m⁻³)

Principle of the Competitive Assay

To quantitatively evaluate the selectivity and binding affinity of nano-MIPs toward insulin, a competitive QCM assay was employed, based on competitive occupation of binding sites between free insulin in solution and insulin immobilized on the QCM surface.

Unlike the direct approach where the concentration of nano-MIPs is varied, here the reverse principle was applied: nanoparticles were introduced into the sensor chamber at a fixed concentration, while the concentration of free insulin in the solution was varied during pre-incubation.

The resulting mixture was then injected into the QCM system, where interaction with surface-immobilized insulin triggered a frequency shift. The higher the fraction of binding sites occupied by insulin in the pre-incubation step, the fewer sites remained available to interact with the immobilized insulin, resulting in a smaller Δf .^{24, 52}

Experimental details of this assay are provided in Sections 2.3.4 and 3.4.4.

1.5 Objectives of the study

The primary objective of this study was to systematically investigate the synthesis, characterization, and performance of molecularly imprinted polymer nanoparticles (nano-MIPs) tailored for selective insulin recognition. The project focused on developing a reproducible synthetic strategy and evaluating the physicochemical and binding characteristics of the obtained nano-MIPs.

Initial synthesis attempts using conventional FRP in aqueous medium revealed insufficient control over particle morphology and consistency. Consequently, the approach was shifted to RAFT polymerization in DMF, which enabled improved control over particle size distribution and batch reproducibility.

Following synthesis, affinity-based separation was employed to isolate high- and low-affinity fractions.

The resulting nanoparticles were subjected to detailed physicochemical characterization. Particle size distribution was determined by DLS, morphology was evaluated by SEM, and surface charge was measured using zeta potential analysis. Fluorescence quenching experiments were used to assess the presence of active binding sites following template removal.

Binding performance was evaluated using QCM measurements on surfaces functionalized either with insulin or with BSA as a non-specific reference. These parallel channels allowed assessment of selective interaction of the nano-MIPs with immobilized insulin.

In addition to optimizing the synthetic process, the study aimed to identify technical challenges and assess consistency across batches. The generated data providing a foundation for further development of nano-MIP systems for biosensing applications involving insulin.

2. Experimental work

2.1 Materials

Silica gel 60 (pore size 60 Å, 70–230 mesh) was obtained from Supelco (Merck, Germany) and used as a solid support. Sodium hydroxide pellets (Honeywell Fluka, ≥98%) were applied for silica activation. For silanization, 3-(triethoxysilyl)propylamine (APTES, Merck, ≥98%) was used, followed by crosslinking with glutaraldehyde (50% aqueous solution, Sigma-Aldrich). Unreacted aldehyde groups were quenched with ethanolamine (≥99%, Sigma-Aldrich).

Human insulin (recombinant, yeast expression) was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and used as the template molecule. Bovine serum albumin (BSA, Fraction V, low heavy metals, Calbiochem) was employed as a non-specific control protein for selectivity studies.

For polymerization, the following monomers were used: N-isopropylacrylamide (NIPAm, Alfa Aesar, 97%), N-tert-butylacrylamide (TBAm, Alfa Aesar, 97%), acrylic acid (AA, Sigma-Aldrich, ≥99%), and N,N'-methylenebisacrylamide (BIS, Alfa Aesar, 99%) as crosslinker. As an additional functional monomer, N-(3-aminopropyl)methacrylamide hydrochloride (APM, Sigma-Aldrich, 90%) was included in selected syntheses. RAFT polymerization was carried out with 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPA, Sigma-Aldrich, ≥97%) as chain transfer agent. For free radical initiation, ammonium persulfate (APS, Sigma-Aldrich, ≥98%) and N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma-Aldrich, ≥99%) were used.

For QCM surface functionalization, additional reagents were employed: 1-butanethiol (n-BuSH, Sigma-Aldrich, ≥99%), ethanolamine (≥99%, Sigma-Aldrich), and cysteamine (Fluorochem, ≥98%).

All solvents were of analytical grade and used without further purification. Dimethylformamide (DMF, anhydrous, ≥99.8%), ethanol (absolute, ≥99.8%), acetone (≥99.5%), and toluene (anhydrous, ≥99.8%) were purchased from Merck/Sigma-Aldrich. Phosphate-buffered saline (PBS, 1× tablets, Biosolute/Th. Geyer GmbH & Co. KG, Germany) was used in all aqueous experiments.

2.2 General procedures for nano-MIP synthesis

2.2.1 Template immobilization and conjugation to analyte

The preparation of insulin-specific nano-MIPs was carried out via a solid-phase approach using silica gel (SG) as the support for template immobilization. Prior to polymerization, the silica surface was chemically activated by boiling SG (15 g) in 1 M NaOH solution (30 mL, 105 °C, 20 min). After thorough rinsing with deionized water, PBS and acetone, the material was dried under vacuum. The activated SG was subsequently silanized with APTES (2.5% v/v in anhydrous toluene, 24 h, room temperature), which introduced primary amino groups on the surface. Following extensive washing with acetone and EtOH/acetone mixtures, the amino-functionalized SG was incubated with 7.5% glutaraldehyde solution in PBS (pH 7.4, 2 h), enabling the formation of aldehyde-functional linkers for covalent attachment of the template. Finally, the SG was conjugated with insulin as the molecular template and thoroughly washed, resulting in an analyte-functionalized support ready for MIP polymerization.

2.2.2 Free-radical polymerization (FRP)

Free-radical polymerization was performed in aqueous medium using ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) as redox initiator system. The polymerization mixture (overall volume of 4 mL, overall monomer concentration of 1.03 mmol/mL) was composed of NIPAm (43 mol-%) as the main backbone monomer, BIS (2 mol-%) as crosslinker, AA (4 mol-%) to introduce carboxyl groups, TBAm (32 mol-%) to modulate hydrophobicity, and APM (19 mol-%) to introduce additional amino functionalities.

Prior to polymerization, all monomers were weighed individually and dissolved in the appropriate solvents (ethanol or Milli-Q water) to prepare concentrated stock solutions. In a typical reaction, 500 μ L of NIPAm stock solution (100 mg/mL), 200 μ L of BIS stock (15 mg/mL), 3 μ L of AA, 500 μ L of TBAm stock (86 mg/mL), and 300 μ L of APM stock (120 mg/mL) were combined in a 15 mL glass vial. The mixture was supplemented with 2.37 mL Milli-Q water to reach a total pre-polymerization volume of ~3.9 mL.

The vial was vortexed thoroughly, followed by sonication for 10 minutes to ensure complete dissolution. To remove dissolved oxygen, which would otherwise inhibit radical propagation, the solution was degassed by argon bubbling for 15 minutes. The insulin-functionalized silica gel was then added as the solid support for imprinting, and the suspension was gently vortexed to ensure homogeneous distribution.

Immediately before initiation, 65 μ L of APS stock solution (60 mg in 240 μ L water) and 65 μ L of TEMED were added. Polymerization was carried out at room temperature for 4 h under gentle agitation. During

this time, the insulin molecules immobilized on the silica surface acted as template for the formation of complementary binding cavities within the growing polymer network.

After completion, the obtained nano-MIPs were separated from the solid phase by affinity-based washing and subsequent elution, as described in Section 2.2.4.

2.2.3 RAFT polymerization

To obtain nano-MIPs containing primary amine functionalities, a monomer mixture comprising NIPAm, BIS, AA, TBAm, and APM was used. The total amount of monomers was 1.03 mmol in a final reaction volume of 4 mL. All syntheses were carried out in DMF, and the polymerization was initiated by a RAFT process using CPA as an iniferter at a concentration of 2.3 mol-%.

The polymerization procedure began with cleaning 15 mL glass vials by sonication in acetone, ethanol, and MilliQ water for five minutes each. The monomers were weighed separately into Eppendorf tubes and dissolved in DMF to prepare stock solutions. Into each glass vial, the following volumes were added: 1.497 mL of DMF, 200 μ L of BIS (15 mg/mL), 500 μ L of NIPAm (100 mg/mL), 3 μ L of AA, 500 μ L of TBAm (86 mg/mL), and 300 μ L of APM (120 mg/mL).

For the nanoMIP batches without APM, the molar proportions of the remaining monomers (NIPAm, BIS, AA, and TBAm) were adjusted to maintain an overall concentration of 1.03 mol-%. For each reaction, 1.556 mL of DMF, 200 μ L of BIS (15 mg/mL), 620 μ L of NIPAm (100 mg/mL), 4 μ L of AA, and 620 μ L of TBAm (86 mg/mL) were added to the glass vial.

The mixtures were vortexed thoroughly and sonicated for 10 minutes to ensure homogeneity, followed by degassing under argon for 15 minutes.

While degassing was ongoing, the iniferter solution (26 mg/mL CPA in DMF) was prepared and vortexed until completely dissolved. After the incubation of the solid phase (silica gel) with the template (insulin or control protein), it was washed with 100 mL of deionized water and added to the polymerization mixture. Subsequently, 1 mL of iniferter solution was added to each vial. Polymerization was initiated by irradiation under a 312 nm UV lamp for two minutes.

2.2.4 Affinity-based separation

After polymerization, the nano-MIPs were released from the insulin-modified solid phase by stepwise elution using an SPE cartridge (15 mL, 100 μ m). The polymerization mixture containing SG and the polymerized material was transferred into the cartridge.

For FRP-derived nano-MIPs, the low-affinity fraction was collected with 50 mL H₂O at 4 °C using a syringe to assist the flow through the cartridge, as the solution did not pass by gravity alone. The high-affinity fraction was then eluted with 50 mL H₂O at 65 °C.

For RAFT-derived nano-MIPs, the same SPE setup was used, but the elution proceeded under gravity flow. The low-affinity fraction was obtained with 50 mL DMF/ACN (50/50) at 4 °C, followed by an early-eluting fraction with 5 mL H₂O at RT, and a high-affinity fraction with 50 mL H₂O at 65 °C.

Collected fractions were cooled to RT and used for downstream characterization.

The yield of each fraction (high- and low-affinity) was determined gravimetrically using 2 mL aliquots. Pre-weighed vials were filled with the respective aliquots, and the solvent was evaporated to dryness by gentle heating. The remaining polymer residue was then weighed, and the obtained mass was converted into ppm. In this work ppm refers to mass concentration and is equivalent to µg/mL. The resulting concentration value was used to estimate the total concentration of the respective fraction.

2.3 Characterization methods

2.3.1 Fluorescence binding assay

Fluorescence spectroscopy (Perkin Elmer LS 50B luminescence spectrometer) was employed to investigate the interaction between insulin and nano-MIPs. All measurements were performed at RT using PBS (pH 7.4) as solvent.

For each measurement, a final assay volume of 2.00 mL was prepared in quartz cuvettes. A 100 ppm insulin solution was mixed with PBS. Then the nano-MIP stock (500 ppm) was added to obtain a final volume of 2 mL, with particle concentrations of 0, 25, 50, 100, 150, 200, and 250 ppm and a constant insulin concentration of 50 ppm. After mixing, spectra were recorded immediately.

Emission spectra were acquired with excitation set at 280 nm, scanning the range of 290–350 nm. Both excitation and emission slit widths were fixed at 4 nm, and the scan speed was maintained at 60 nm/min. Control spectra of insulin alone and PBS buffer were recorded under identical conditions.

For ease of comparison all spectra were normalized to the maximum fluorescence intensity of insulin in the absence of nano-MIPs. The resulting overlays demonstrated a progressive decrease in fluorescence intensity with increasing nano-MIP concentration, reflecting the quenching effect associated with insulin binding.

2.3.2 Dynamic Light Scattering (DLS) and zeta potential

The particle size and surface charge of the nano-MIPs were measured with a Zetasizer Nano ZS (Malvern Panalytical, UK). For these measurements, only the high-affinity fractions obtained after affinity separation were used. The suspensions were concentrated to 2000 ppm and measured in deionized water without any additional buffer.

Immediately before measurement, samples were passed through 200 µm syringe filters. No sonication was applied. All experiments were carried out at 20 °C.

DLS measurements were performed in three runs averaging 10 measurements each, while for the zeta potential, 3 runs averaging with 10-15 measurements per run were recorded. Disposable cuvettes were used for size analysis, and folded capillary cells (DTS1070, Malvern) were used for zeta potential measurements.

2.3.3 Scanning electron microscopy (SEM)

The morphology of the nano-MIPs was examined using a field-emission scanning electron microscope (FE-SEM, Zeiss Supra 55VP). For sample preparation, silicon wafer chips (5 × 5 mm) were cleaned by sequential sonication in acetone and ethanol (5 min each). The cleaned substrates were fixed onto SEM stubs and sputter-coated with a thin conductive layer of gold (5 nm).

After affinity separation, both the low- and high-affinity nano-MIP fractions were deposited onto the prepared substrates and dried overnight at room temperature to ensure complete solvent evaporation. The samples were then analyzed under high-vacuum conditions. Representative SEM micrographs were acquired at different magnifications in order to evaluate the morphology, surface texture, and size distribution of the nanoparticles. The measurements were performed at an accelerating voltage (EHT) of 5 kV.

2.3.4 Quartz Crystal Microbalance measurements (QCM)

Preparation of QCM electrodes and measurement setup.

Quartz crystal microbalance (QCM) sensors were prepared from 10 MHz AT-cut quartz blanks (Ø 13.8 mm). For the fully metallized measurement side of the quartz, a thin chromium layer (around 5 nm) was first deposited by physical vapor deposition, followed by evaporation of a 30 nm gold layer. Screen-printing of colloidal gold paste (Heraeus 10% brilliant gold paste) was employed to form the circular electrodes (Ø ~5 mm) on the back side of the crystal. The electrodes were subsequently cured at 400 °C for 4 h, resulting in mechanically stable chromium/gold layers suitable for QCM operation. The prepared QCM are shown in **Figure 2.1**.

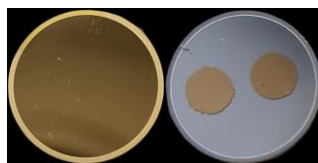


Figure 2. 1: Gold-coated quartz crystals prepared with chromium/gold electrodes prior to functionalization.

For QCM experiments, the functionalized chips were mounted in a custom-built flow cell equipped with inlet/outlet tubing (**Figure 2.2**) and contacted via SMA connectors on the backside (**Figure 2.3**). The resonance behavior of each electrode was first verified using a network analyzer (Agilent/Keysight

E5062A). Each chip displayed a single, stable resonance within the 9.87-9.94 MHz range, with the exact value depending on surface conditions and functionalization.

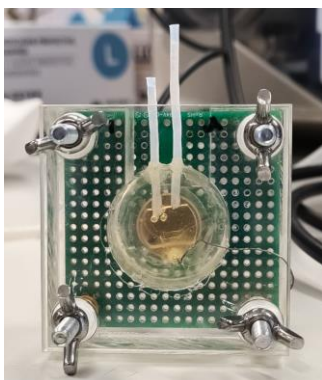


Figure 2. 3: Flow cell, front view.

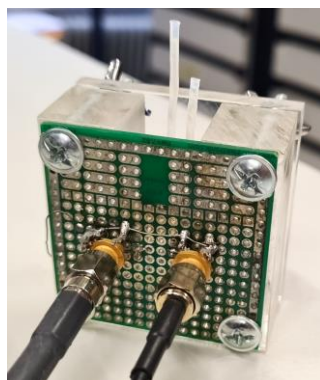


Figure 2. 2: Backside electrical contacts with SMA connectors.

During measurements, the crystal was driven by a laboratory power supply (EA-PS 2032-025), operated in the range of 14.9-15.2 V and 0.05-0.07 A. Frequency shifts (Δf) were monitored in real time using a frequency counter (Agilent 53131A) and logged via the PC interface (**Figure 2.4**). Milli-Q water was used as the carrier and baseline medium. After establishing a stable baseline, suspensions of nano-MIPs prepared in Milli-Q water were manually introduced into the flow cell. The resulting Δf responses were recorded, and steady-state plateaus were taken as representative binding signals.

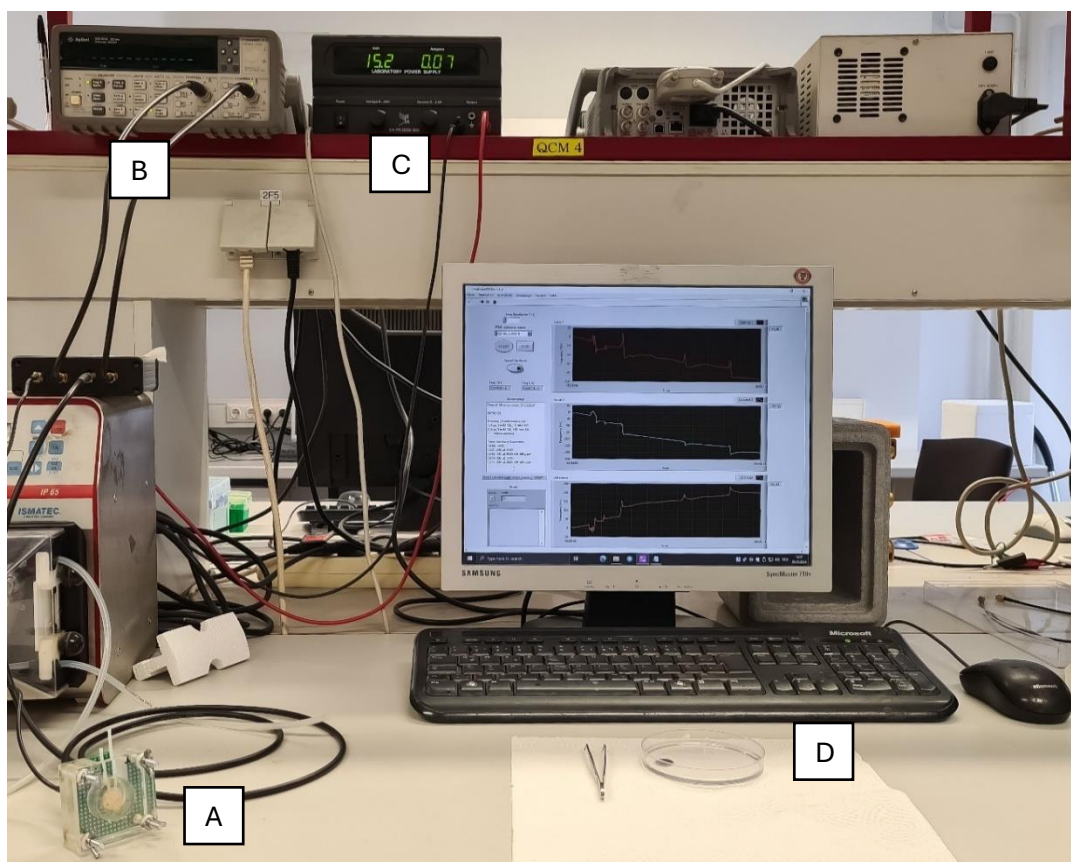


Figure 2. 4: QCM measurement setup. A - custom flow cell with mounted quartz crystal; B - frequency counter (Agilent 53131A); C - laboratory power supply (EA-PS 2032-025); D - PC interface for data logging.

2.3.5 Electrode functionalization protocols

Substrate cleaning

Gold-coated 10 MHz AT-cut quartz crystals were rinsed with acetone and ethanol (~30 s each), dried under N₂ and treated in a plasma cleaner (Diener Zepto) for 15 min.

Protocol A - Cysteamine only without spacer (Figure 2.5).

Chips were covered with a cysteamine solution (2 mM) in ethanol in sealed Petri dishes and left overnight at RT. After rinsing with ethanol and PBS and drying in air, the surfaces were activated with glutaraldehyde (GA, 5 mM in PBS, 2 h, RT) and washed (EtOH → PBS).

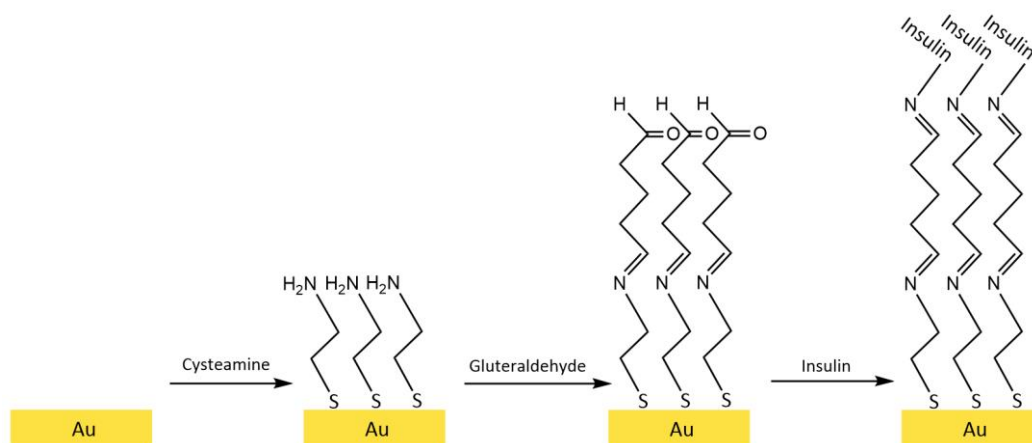


Figure 2. 5: Protocol A functionalization scheme

Protocol B - Cysteamine with the spacer 1-butanethiol (Figure 2.6).

To reduce the surface density of amines, a mixed monolayer was formed from cysteamine (2 mM in ethanol) and 1-butanethiol (1000 mM, corresponding to a 1:500 Cys:Bu ratio). The solution was prepared and handled in a fume hood. Chips were incubated with this mixture overnight at RT, then rinsed (EtOH → PBS), reacted with GA 5 mM in PBS for 2 h, and washed as above.

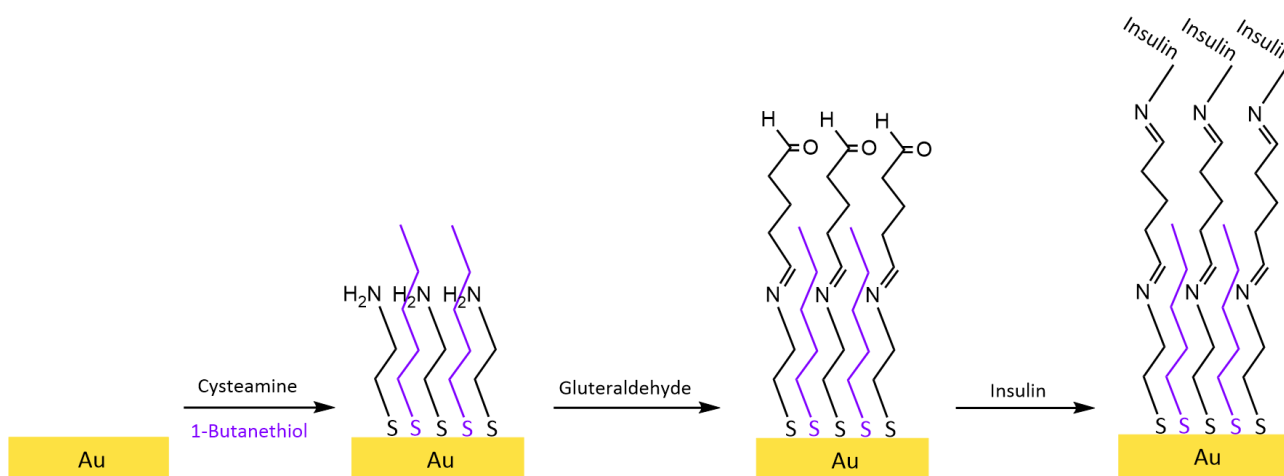


Figure 2. 6: Protocol B functionalization scheme

Protein immobilization and blocking

After GA activation, 5-10 μL droplets were spotted onto each gold electrode: insulin (6 mM in PBS) or BSA (500 ppm in PBS) as a control (**Figure 2.7**). Chips were incubated overnight in sealed dishes to prevent evaporation. Where indicated, residual aldehydes were quenched with ethanolamine (6 mM in PBS, 20-30 min), followed by PBS rinse and air-drying (**Figure 2.8**).

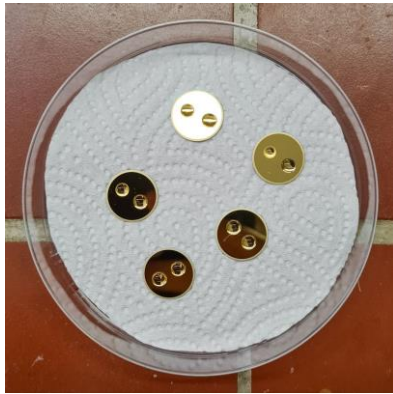


Figure 2. 7: Spotting of insulin and BSA droplets onto gold electrodes.

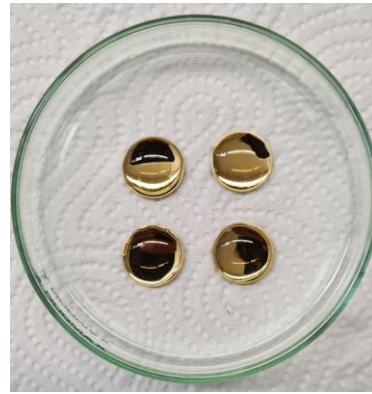


Figure 2. 8: Gold electrodes after ethanolamine incubation.

3. Results and Discussion

3.1 The optimization of the solid-phase synthesis

The experimental work presented in this study was focused on the systematic optimization of a solid-phase synthesis protocol for insulin-specific nano-MIPs. Initially, nano-MIPs were synthesized using classical free radical polymerization in aqueous medium (EE03), with APM incorporated as a functional monomer. Although polymerization was successful, the resulting particles exhibited considerable heterogeneity in physicochemical properties, particularly regarding size distribution and morphology.

To improve control over particle formation and enhance reproducibility, RAFT polymerization was introduced starting from batch EE02, using DMF as the reaction solvent. However, the presence of APM was maintained in EE02, and its influence on particle uniformity and nonspecific binding was still observed.

Subsequently, in batches EE04 to EE06, RAFT polymerization in DMF was continued with the exclusion of APM from the monomer mixture. This adjustment led to improved particle homogeneity and colloidal stability.

Affinity separation was consistently performed after each polymerization step to isolate high-affinity fractions. The purified nano-MIPs were then subjected to characterization, including fluorescence-based binding assays for affinity evaluation, DLS and zeta potential measurements for particle size and surface charge analysis, SEM for morphological assessment, and QCM measurements for real-time binding studies with immobilized insulin on gold-coated sensor surfaces.

3.2 Nano-MIP synthesis via FRP

In the initial phase of this study, nano-MIPs were synthesized via classical free radical polymerization conducted in aqueous medium.

The evaluation of the aqueous polymerization approach is based on the results from batch EE03. Affinity separation of the nano-MIP dispersion obtained from batch EE03 yielded a high-affinity (HA) fraction with a concentration of 183 ppm and a low-affinity (LA) fraction with a concentration of 1100 ppm.

SEM imaging of the HA fraction revealed the presence of aggregated structures (**Figure 3.1**). Although aggregation was prominent, individual nano-sized spherical particles (~60 nm) could still be distinguished within the clusters, suggesting the successful formation of nano-MIP structures. In contrast, the LA fraction exhibited a very different morphology (**Figure 3.2**), characterized by the presence of needle-like structures and irregular polymeric debris. These features are typically associated with unreacted or partially crosslinked monomer residues, indicating the non-specific nature of the LA fraction.

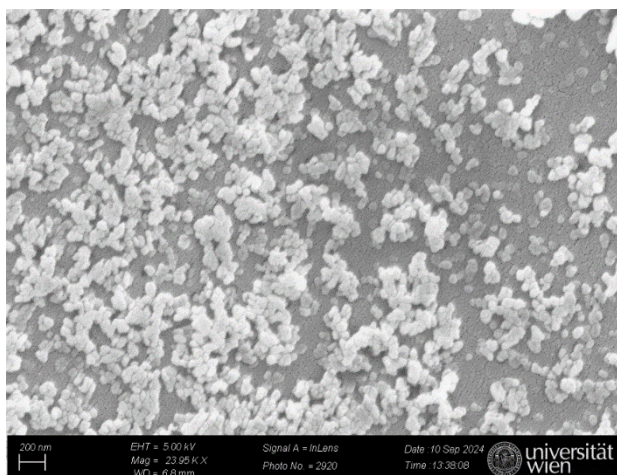


Figure 3. 1: SEM image of the HA fraction (500 ppm) obtained from batch EE03. Scale bar = 200 nm.

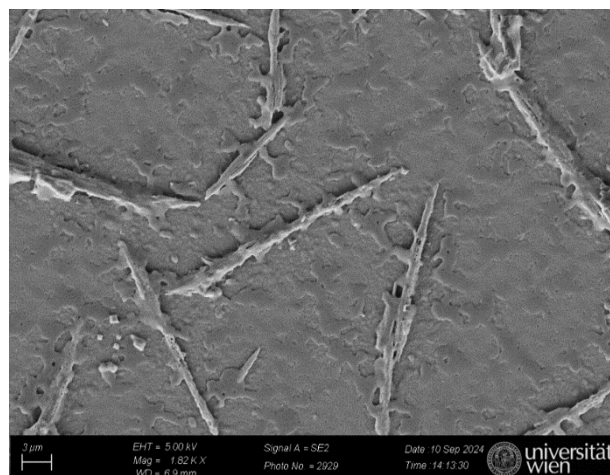


Figure 3. 2: SEM image of the LA fraction (1100 ppm) obtained from batch EE03. Scale bar = 3 μm.

These features are typically associated with unreacted or partially crosslinked monomer residues, indicating the poor quality and non-specific nature of the LA fraction.

Fluorescence-based binding assays were performed using a constant insulin concentration of 50 ppm, while the concentration of nano-MIPs was varied (0 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm and 250 ppm). As shown in **Figure 3.3**, the fluorescence intensity showed only minimal quenching upon increasing nano-MIP concentrations. The decrease in fluorescence was weak and inconsistent, suggesting that the insulin molecules were not effectively bound or shielded by the nano-MIPs. The emission maximum was observed at 307.5 nm, and this value was used as a reference point to assess fluorescence quenching.

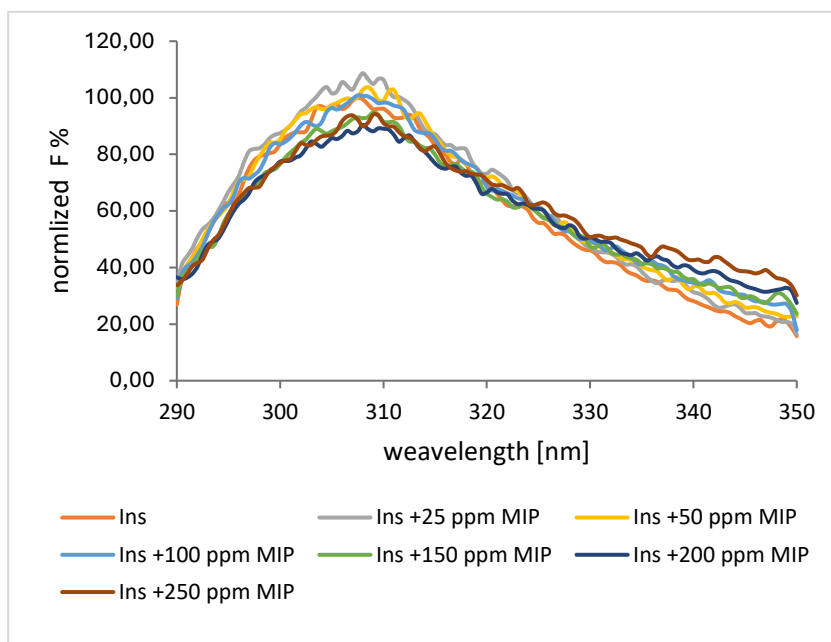


Figure 3. 3: Normalized fluorescence spectra of insulin solutions upon incubation with increasing concentrations of HA nano-MIPs (batch EE03).

The weak quenching effect could potentially be related to the aggregation of nano-MIPs observed in the SEM images, which might have restricted the accessibility of the recognition sites and thus impaired binding interactions with insulin.

Together, these results suggest that classical free radical polymerization in aqueous medium, under the applied conditions, did not provide sufficient control over particle morphology and binding site accessibility, leading to the necessity for an alternative polymerization approach with improved regulation of particle formation.

3.3 Nano-MIP synthesis via RAFT polymerization in DMF with APM

In the next phase of this study, the polymerization strategy was adapted to employ RAFT polymerization, aiming to improve the control over particle nucleation and growth. Batch EE02 was synthesized using RAFT polymerization in DMF as the reaction solvent. The monomer composition included NIPAm, BIS, acrylic acid and TBAm as functional monomers, and APM for introducing amino groups. The concentrations of the obtained fractions were determined and yielded for HA fraction 450 ppm and for LA fraction 1100 ppm.

The HA fraction in EE02 was initially below the target analytical concentration and was therefore adjusted to 500 ppm for fluorescence binding assays and 2000 ppm for DLS and zeta potential measurements by mild solvent evaporation.

3.3.1 Fluorescence binding assays

The normalized fluorescence spectra shown in **Figure 3.4** demonstrate a progressive quenching effect upon increasing nano-MIP concentrations. The fluorescence intensity decreased systematically, indicating the occurrence of binding events between the nano-MIPs and insulin molecules. At the highest nano-MIP concentration (250 ppm), a reduction of fluorescence intensity by approximately 40% was observed, suggesting moderate binding interactions. The fluorescence maximum of insulin was located at 310 nm, and quenching percentages were evaluated relative to this wavelength. The progressive quenching profile implies that accessible recognition sites are present on the surface of the nano-MIPs, allowing insulin to interact effectively. However, the incomplete quenching as compared to an ideal case of strong binding leading to nearly complete fluorescence suppression suggests that not all binding sites were fully accessible or fully functional.

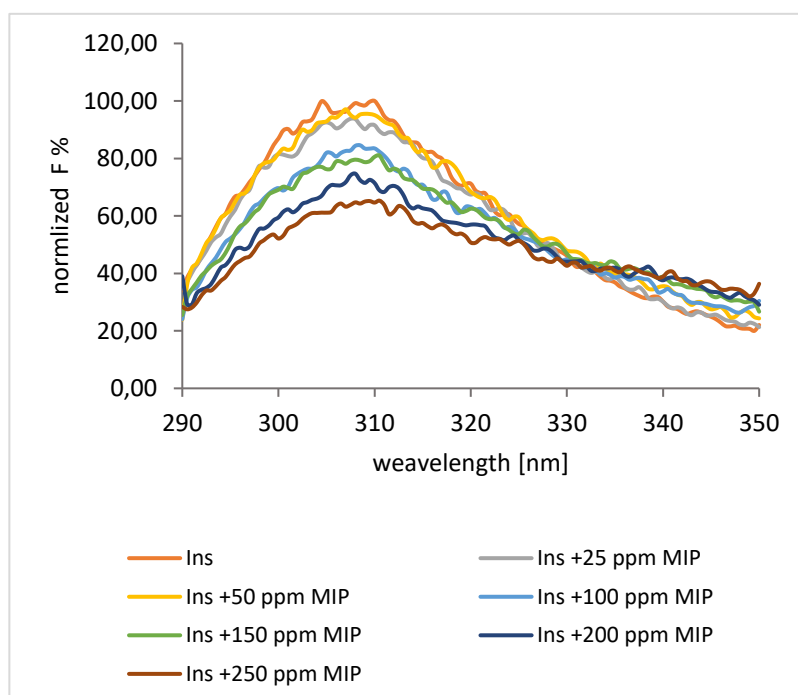


Figure 3. 4: Normalized fluorescence spectra of insulin solutions upon incubation with increasing concentrations of HA nano-MIPs (batch EE02).

Possible reasons for the moderate quenching effect include the partial aggregation of nano-MIPs, limiting the surface area available for interaction, and the potential inefficiency of the imprinting process during polymerization. Residual template molecules, suboptimal template removal, or imperfections in cavity formation could contribute to reduced binding efficiency.

Thus, fluorescence data suggests that while RAFT polymerization with APM in DMF improved the overall binding behavior compared to free radical polymerization in aqueous media, further optimization was still necessary to enhance binding site accessibility and binding strength.

3.3.2 Dynamic Light Scattering and Zeta Potential Measurements

DLS was employed to characterize the size distribution and colloidal behavior of the HA nano-MIP fraction. The average hydrodynamic diameter of the EE02 fraction was measured to be 200 ± 24 nm with a PDI of 0.30 ± 0.04 , indicating a moderate degree of size heterogeneity (**Figure 3.5**).

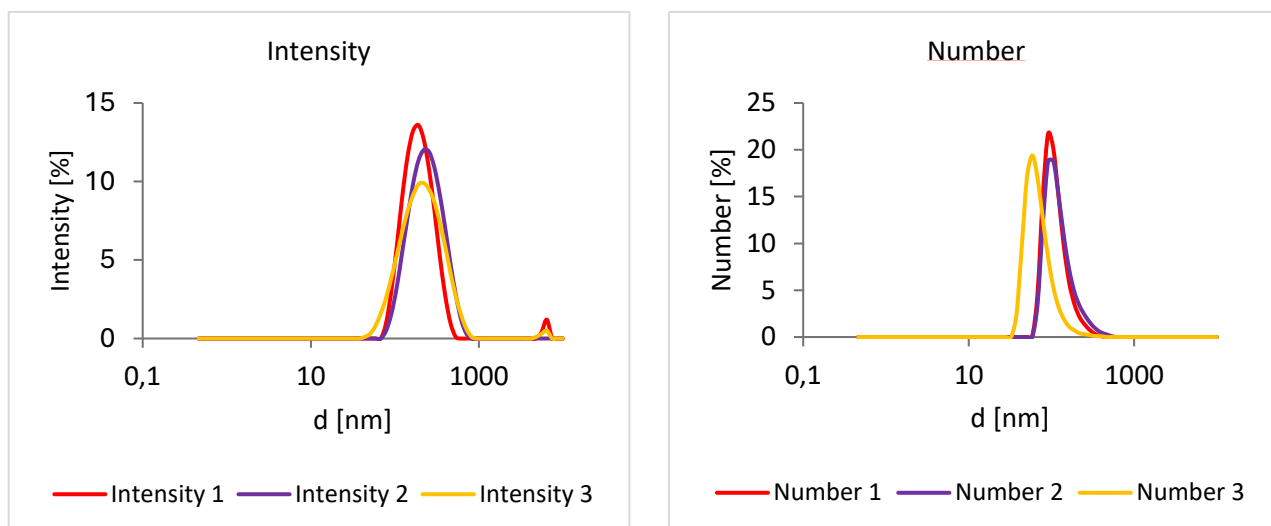


Figure 3. 5: DLS analysis of the HA fraction from batch EE02: (**left**) particle size distribution by intensity; (**right**) particle size distribution by number.

The intensity-weighted size distribution (**Figure 3.5, left**) reveals a predominant peak centered around 200-300 nm. In addition to the main population, a minor secondary peak was observed at sizes greater than 1000 nm, accounting for a small fraction of the total intensity. The presence of this secondary peak indicates the existence of a low amount of larger aggregates within the dispersion. Although the aggregates represent a small numerical fraction, their contribution to the scattering intensity is disproportionately large because larger particles scatter light much more strongly than smaller ones.

The number-weighted size distribution (**Figure 3.5, right**) provides complementary information by displaying the percentage of total particles associated with different size ranges. The main peak was centered at approximately 80-100 nm, reaching a maximum of about 22-23% of the particle count. This indicates that the majority of particles in the dispersion were in the nano-size range, consistent with the expected characteristics of nano-MIPs.

Notably, no significant population was observed in the size range above 1000 nm in the number distribution, supporting the conclusion that aggregation was present only to a minor extent and did not dominate the particle population numerically.

These findings are corroborated by the measured PDI of 0.30, which indicates a moderate degree of size heterogeneity. Although the sample demonstrated a reasonable level of monodispersity, the presence of minor aggregation must be taken into account when interpreting the binding performance of the nano-MIPs.

Zeta potential measurements yielded a value of $+25 \pm 2$ mV, reflecting a moderately positive surface charge imparted by the amino groups introduced via APM. This level of surface charge is typically sufficient to confer moderate colloidal stability in polar solvents such as DMF. However, the detection of aggregation in the intensity plots suggests that the repulsive electrostatic forces were not fully effective in preventing particle-particle interactions, possibly due to the nature of the DMF environment or local heterogeneities in surface charge density.

Together, DLS and zeta potential data indicate that RAFT polymerization with APM led to significant improvements in nano-MIP particle size control compared to classical polymerization in water, but that challenges related to colloidal stability and aggregate formation still remained.

These findings motivated the subsequent optimization steps, specifically the exclusion of APM from the polymerization mixture in following batches (EE04 onwards) to assess its impact on particle homogeneity and binding performance.

3.4 Nano-MIP synthesis via RAFT polymerization in DMF without APM

In the subsequent experimental phase, nano-MIPs were synthesized via RAFT polymerization in DMF without the incorporation of APM using the same procedure (batches EE04, EE05 and EE06). This modification aimed to further improve particle homogeneity, reduce nonspecific interactions, and enhance the binding site fidelity, based on observations from earlier batches incorporating APM.

To comprehensively characterize the structural and functional properties of the resulting nano-MIPs, a set of analytical techniques was employed. These included SEM for morphological analysis, DLS and zeta potential measurements for particle size and surface charge evaluation, fluorescence-based binding assays to assess the affinity towards insulin, and QCM measurements for real-time label-free interaction studies.

Additionally, a competitive binding assay was performed for the HA fraction obtained from batch EE06 to further evaluate the selectivity of the synthesized nano-MIPs. This assay provides critical information on the specificity of the binding interaction by assessing the ability of free insulin molecules to compete with immobilized analytes for the binding sites on the nano-MIPs.

The results obtained from these characterization methods are presented and discussed in the following sections.

3.4.1 Morphological characterization (SEM)

The morphological features of the high- and low-affinity nano-MIP fractions from batches EE04, EE05, and EE06 were analyzed using scanning electron microscopy. Representative SEM images are shown below.

Batch EE04

The concentrations of the high-affinity (400 ppm) and low-affinity (150 ppm) fractions were determined from batch EE04. Due to the limited concentration of the HA fraction, the samples were concentrated prior to further analysis. For fluorescence measurements, the final working concentration was adjusted.

SEM analysis of the HA fraction (400 ppm) reveals discrete nano-sized spherical particles with slightly textured surfaces (**Figure 3.6A**). The particles appeared individually separated with minor aggregation. The background exhibited minimal amorphous material, suggesting a relatively clean separation of the nano-MIPs after synthesis.

The LA fraction (150 ppm) exhibited a markedly different morphology, characterized by larger, irregularly shaped aggregates embedded in an amorphous matrix (**Figure 3.6B**). The presence of such structures suggests incomplete polymerization and the presence of non-specific material, which is typical for low-affinity fractions.

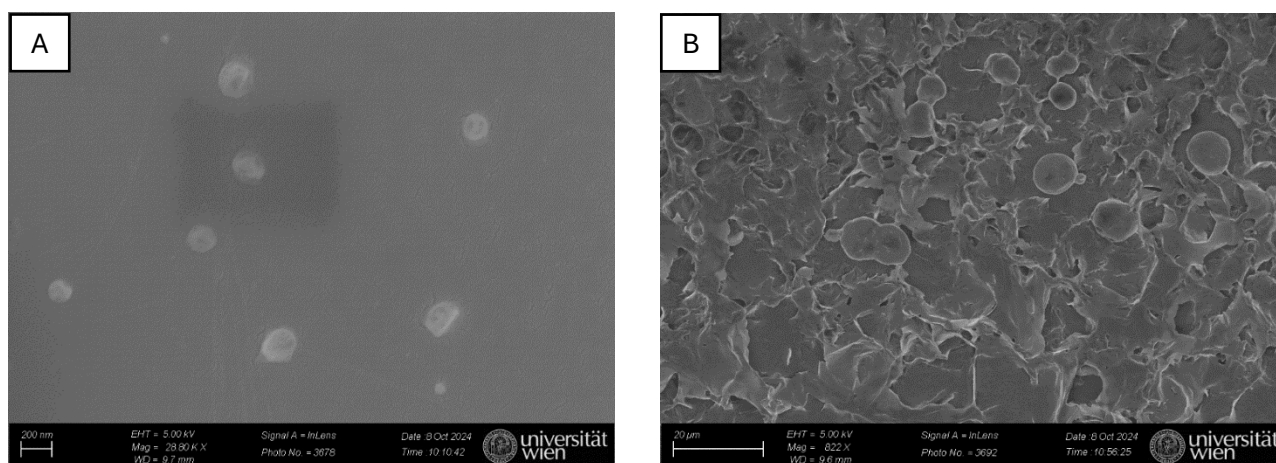


Figure 3. 6: SEM images of the HA (400 ppm) and LA (150 ppm) fractions from batch EE04, showing spherical nano-MIPs with textured surfaces (**A**) and fibrous amorphous structures with embedded aggregates (**B**).

Batch EE05

For batch EE05, high and low-affinity fractions were similarly isolated following affinity separation procedures. The measured concentrations of each fraction are 500 ppm for HA and 400 ppm for LA.

While the HA fraction reached 500 ppm after affinity separation and was directly suitable for fluorescence measurements, the sample was further concentrated to 2000 ppm for DLS and surface charge characterization..

SEM images of the HA fraction revealed a well-defined population of spherical nano-sized particles (**Figure 3.7A**). The particles exhibit relatively uniform morphology and appeared individually dispersed across the substrate, with minimal aggregation. Compared to EE04, the improved dispersion and clearer particle definition indicates a more homogeneous polymerization process and improved colloidal stability in EE05.

In contrast, the LA fraction predominantly exhibited fibrous, network-like structures (**Figure 3.7B**). These morphologies are consistent with loosely crosslinked or unstructured polymer material typically removed during washing steps. No defined spherical nano-MIPs are visible in the LA fraction, supporting the conclusion that this material consisted of non-specific polymeric debris.

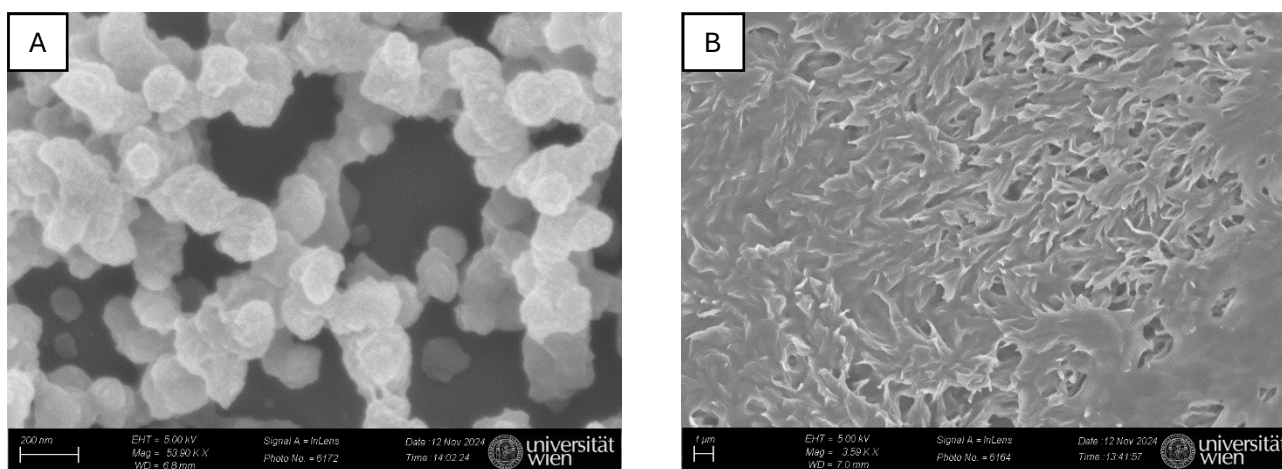


Figure 3. 7: SEM images of the HA (500 ppm) and LA (400 ppm) fractions from batch EE05, showing spherical nano-MIPs with textured surfaces (**A**) and fibrous amorphous structures with embedded aggregates (**B**).

Batch EE06

Affinity separation of batch EE06 yielded high- and low-affinity fractions with measured concentrations of 150 ppm and 50 ppm, respectively.

Due to the low yield of material in this batch, the HA and LA fractions were concentrated prior to further analysis. For fluorescence-based binding studies, the samples were adjusted to 500 ppm, while for DLS and surface charge (zeta potential) measurements, the concentrations increased to 2000 ppm. The concentration process was performed by gentle solvent evaporation on a heating plate at moderate temperature.

SEM imaging of the HA fraction (**Figure 3.8A**) shows moderately aggregated spherical structures, with partial surface irregularities. Compared to EE05, the particles were less individually resolved, and some morphological softening was visible. This may reflect the lower initial material concentration or changes in surface properties due to the concentration step.

The LA fraction (**Figure 3.8B**) was characterized by an amorphous, fibrous matrix, with no clearly defined nanoparticles observed. This morphology is typical of unstructured polymeric residues removed during the washing phase, and the extremely low concentration in this fraction may have further limited morphological clarity during imaging.

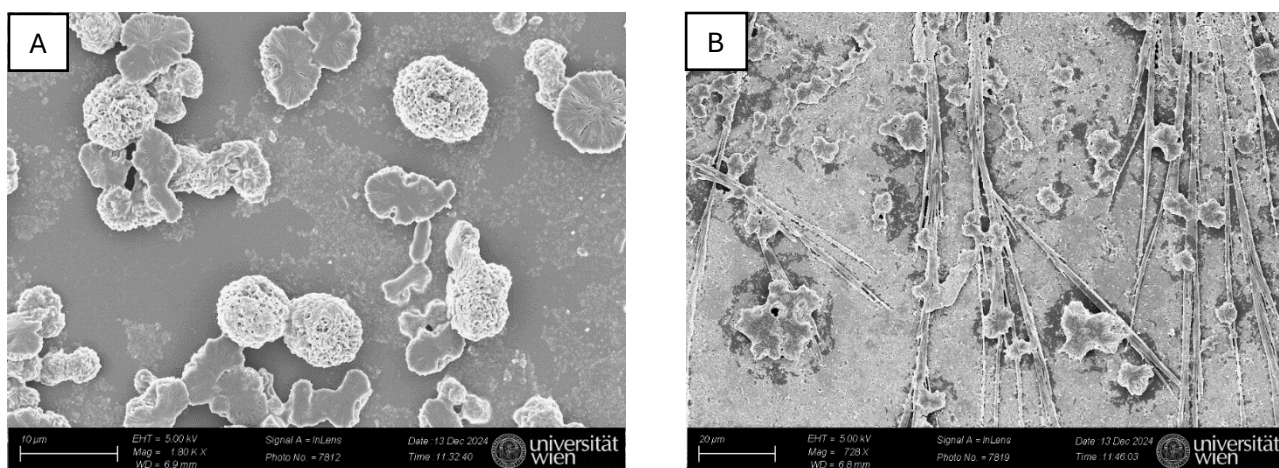


Figure 3. 8: SEM images of the HA (500 ppm) and LA (50 ppm) fractions from batch EE05, showing spherical nano-MIPs with textured surfaces (**A**) and fibrous amorphous structures with embedded aggregates (**B**).

3.4.2 Particle size (DLS) and surface charge analysis (zeta potential)

Batch EE04

The measured DLS and zeta potential parameters for the HA fraction displays an average diameter of 140 ± 2 nm and a PDI of 0.30 ± 0.00 , indicating moderate heterogeneity. The size by number was 14 ± 2 nm, and the zeta potential was measured at -42 ± 2 mV, which reflects strong surface charge and supports good colloidal stability.

The intensity distribution (**Figure 3.9, left**) revealed a clearly bimodal pattern, with a primary peak centered around 140-170 nm, corresponding to the expected nano-MIP size range, and a secondary shoulder in the 10-30 nm range. The latter was consistently observed across all replicates and may represent residual polymer fragments, very small polymer species or incomplete particles that were not fully removed during the separation process.

In contrast, the number-based distribution (**Figure 3.9, right**) displayed a highly monodisperse peak at ~ 15 nm, reflecting the numerical dominance of smaller particles, even though they contribute relatively little to total scattering intensity. The sharpness of this peak indicates good reproducibility and size homogeneity at the lower end of the size spectrum.

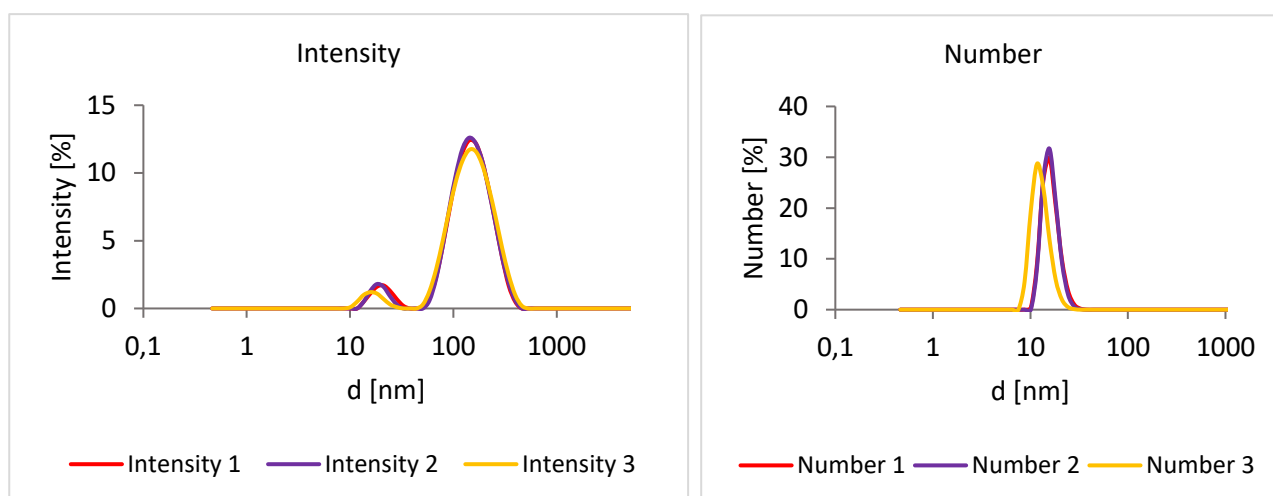


Figure 3. 9: DLS analysis of the HA fraction from batch EE02: (**left**) particle size distribution by intensity; (**right**) particle size distribution by number.

No aggregation peaks were observed above 1000 nm in either distribution, confirming that the colloidal system remained stable after concentration. Together, the DLS and zeta potential data indicate a well-dispersed particle population with moderate heterogeneity and electrostatic stabilization.

Batch EE05

The particles the HA fraction exhibited an average diameter of 25 ± 1 nm and an identical PDI of 0.30 ± 0.13 , as observed in EE04. Despite the numerical similarity in polydispersity, the particle population in EE05 was notably smaller and more tightly distributed. The number-based size was measured at 16 ± 0 nm, and the zeta potential was -31 ± 2 mV, confirming a stable colloidal system with sufficient electrostatic repulsion.

The intensity distribution (**Figure 3.10A**) revealed a main peak centered around 25-30 nm, which corresponds well to the size intensity reported in the table. Additionally, a secondary broader peak was observed between 200 and 300 nm, along with a low-intensity but reproducible signal near 8000-9000 nm, suggesting the presence of a small fraction of aggregated material. While this signal is not reflected in the number distribution, it likely corresponds to a negligible amount of large aggregates, optically visible due to their scattering properties. This may indicate the onset of time-dependent aggregation, as the measurement was not performed immediately after synthesis.

In contrast, the number distribution (**Figure 3.10B**) showed a narrow and symmetric peak at approximately 15-20 nm, with no indication of higher size species, confirming that the main particle population remained well-dispersed by count.

Taken together, the results indicate that EE05 produced a relatively small, homogeneous nano-MIP population with minor aggregation effects, likely related to storage, but overall retained excellent dispersion and colloidal behavior.

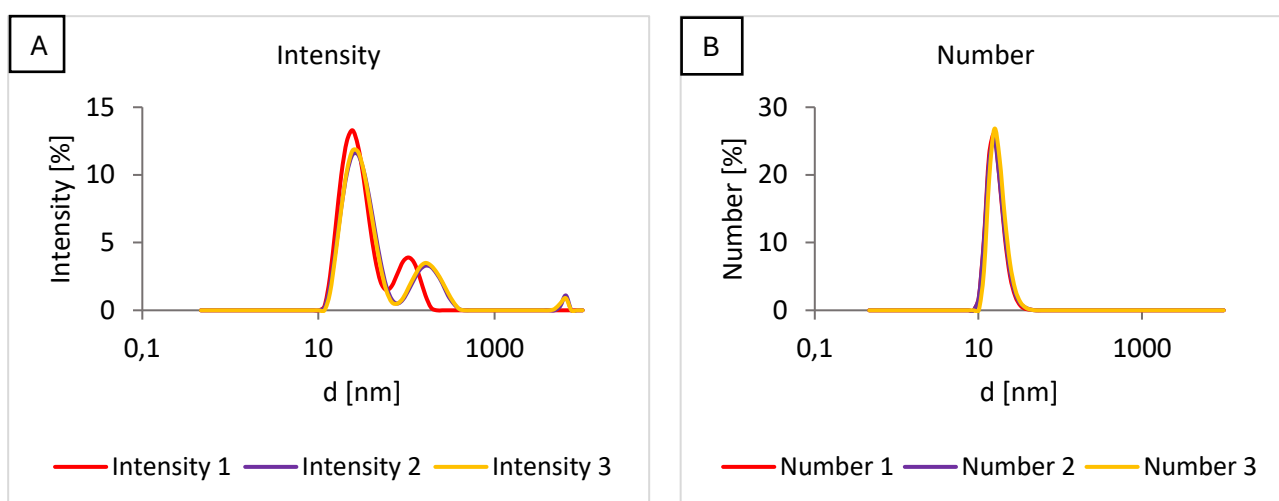


Figure 3. 10: DLS measurements for the HA fraction of batch EE05 at 2000 ppm.

(A) Intensity-weighted size distribution.

(B) Number-weighted size distribution.

Batch EE06

The measured average diameter for the high-affinity fraction was 32 ± 1 nm and a number-based size of 19 ± 2 nm, indicating a tightly packed nano-MIP population. The PDI was 0.20 ± 0.00 , the lowest among all batches, reflecting a highly monodisperse system. The zeta potential was -27 ± 2 mV, which falls within the range for electrostatically stable colloids, though slightly lower than that observed in previous batches.

The intensity-weighted size distribution (**Figure 3.11A**) shows a dominant, narrow peak centered around 30 nm, which was highly consistent across replicates. A broad, low-intensity region spanning from ~600 to ~7000 nm was also detected in all measurements, indicating the presence of a minor population of larger particles or aggregates. While this aggregation was minimal, its reproducibility suggests possible early-stage clustering, potentially influenced by storage conditions.

The number distribution (**Figure 3.11B**) confirmed the monodisperse nature of the primary population, with all replicates displaying a sharp peak around 20 nm. While the peaks did not fully coincide, their close alignment and consistent shape indicate only minor inter-replicate variation, further supporting the uniformity of the particle system.

Overall, the HA fraction of EE06 exhibited good colloidal characteristics, with a tight size distribution and only minimal signs of aggregation, representing the most uniform nano-MIP population obtained among all synthesized batches.

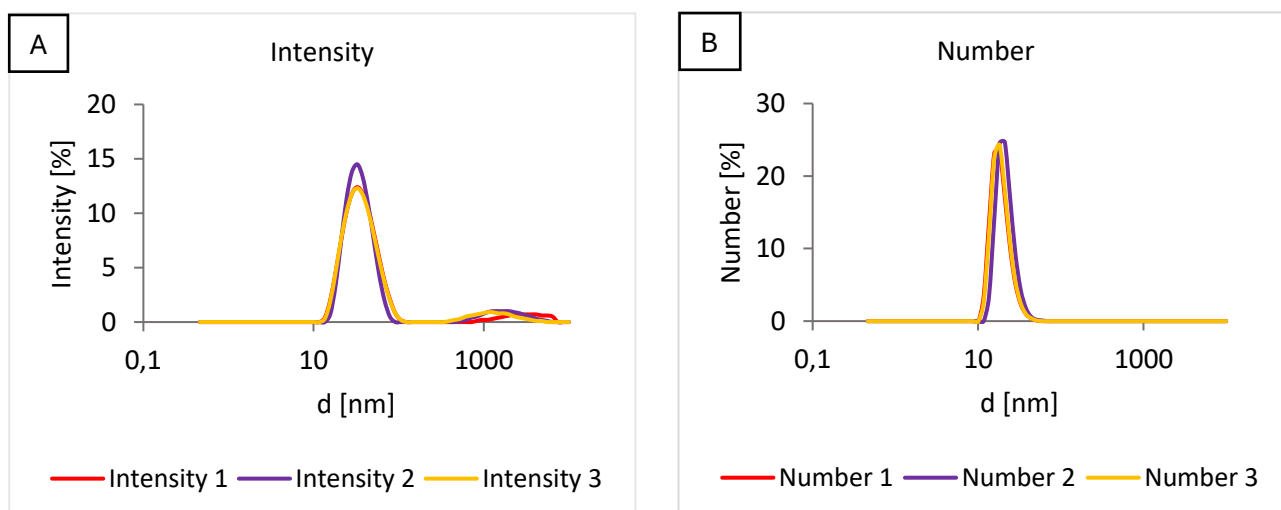


Figure 3. 11: DLS measurements for the HA fraction of batch EE06 at 2000 ppm.

(A) Intensity-weighted size distribution.

(B) Number-weighted size distribution.

To provide a clear overview of the physicochemical characteristics of all synthesized nano-MIP batches, a comparative summary is presented in **Table 3.1**. Data are presented as average $\pm$ standard deviation for the HA fractions, except for yield values, which are shown for both HA and LA fractions.

Table 3. 1: Summary of synthesized batches characteristics, including yield, hydrodynamic size, zeta potential, and PDI values.

Batch	HA yield [ppm]	LA yield [ppm]	Size Intensity [nm]	Size by number [nm]	Zeta Potential [mV]	PDI
EE02	450	1100	200 $\pm$ 24	80 $\pm$ 19	+25 $\pm$ 2	0.30 $\pm$ 0.04
EE04	400	150	140 $\pm$ 2	14 $\pm$ 2	-42 $\pm$ 2	0.30 $\pm$ 0.00
EE05	550	400	25 $\pm$ 1	16 $\pm$ 0	-31 $\pm$ 2	0.30 $\pm$ 0.13
EE06	150	50	32 $\pm$ 1	19 $\pm$ 2	-27 $\pm$ 2	0.20 $\pm$ 0.00

The presented data clearly show that the presence of APM in the monomer mixture has a significant impact on the properties of the resulting nanoparticles. Batch EE02, synthesized with APM, exhibited a positive ζ -potential (+25 mV), whereas all other RAFT batches (EE04–EE06), where APM was omitted, showed negative values ranging from -27 to -42 mV. This shift in surface charge can be directly attributed to the incorporation of primary amine groups from APM, which are partially protonated at neutral pH, resulting in a net positive charge. Additionally, EE02 displayed the largest hydrodynamic diameter (around 200 nm by intensity), which contrasts with the smaller sizes observed in the other batches. This may be explained by potential aggregation effects or might be related to the presence of APM, but further studies would be required to confirm any causal relationship.

3.4.3 Binding affinity evaluation (Fluorescence assay)

Batch EE04

A clear and gradual quenching of insulin fluorescence is observed with increasing nano-MIP concentration (**Figure 3.12**), indicating the formation of binding interactions between the particles and the target molecule. At the highest tested concentration (250 ppm), the fluorescence intensity was reduced by approximately 60%, demonstrating a moderate but significant quenching response. The fluorescence intensity was evaluated at 307.5 nm, corresponding to the emission maximum of the insulin signal in this batch.

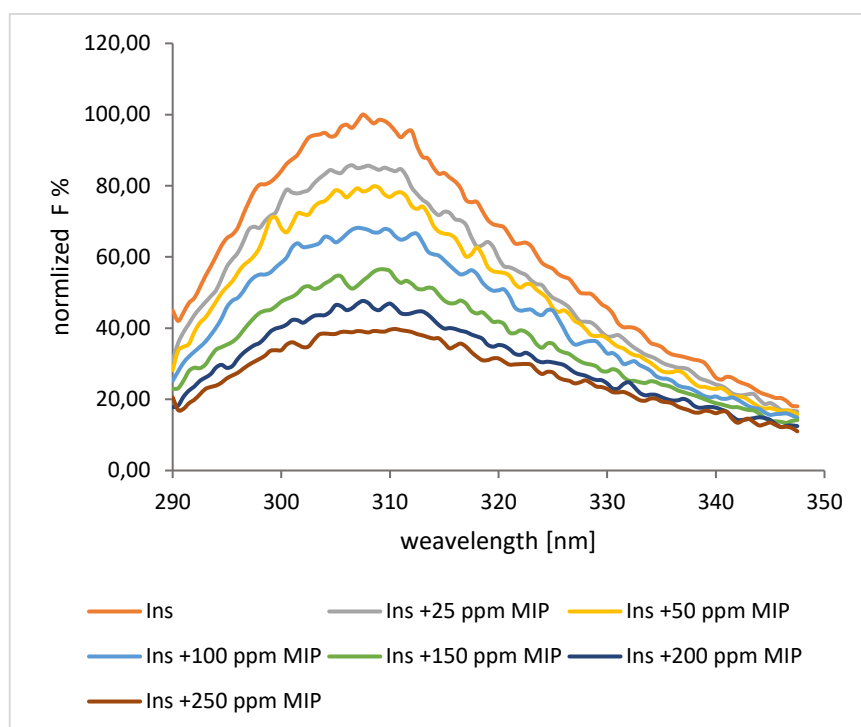


Figure 3. 12: Normalized fluorescence spectra of insulin (50 ppm) with increasing concentrations of HA nano-MIPs from batch EE04 (25–250 ppm).

The fluorescence quenching profile remained smooth and uniform over the entire range of concentrations investigated. No signs of saturation or sharp jumps were observed. This characteristic of the curve suggests that there are indeed available recognition sites on the surface of nano-MIPs that can efficiently interact with insulin. At the same time, the fluorescence signal recorded at 250 ppm shows that some insulin remained free in solution.

It is also possible that some structural constraints in the imprinted cavities also influence this behavior. In addition, a batch of EE04 was synthesized without the addition of APM, which means that there are no positively charged functional groups in the polymer structure, which can enhance electrostatic interactions with the, at neutral pH, negatively charged insulin molecules. The relatively homogeneous particle size distribution observed by DLS data, as well as the stable zeta potential, confirm that the

obtained MIP-particles are functional, stable in colloidal form and show pronounced binding characteristics.

Batch EE05

As **Figure 3.13** shows, addition of nano-MIPs leads to a systematic decrease in fluorescence intensity, indicating the presence of interaction between imprinted particles and insulin molecules. However, the degree of quenching was relatively low: at 250 ppm, the signal intensity in the 308.5 nm region was reduced by about 45%. In comparison, in the EE04 batch, the quenching reached about 60 %, indicating denser and more specific binding in the previous case.

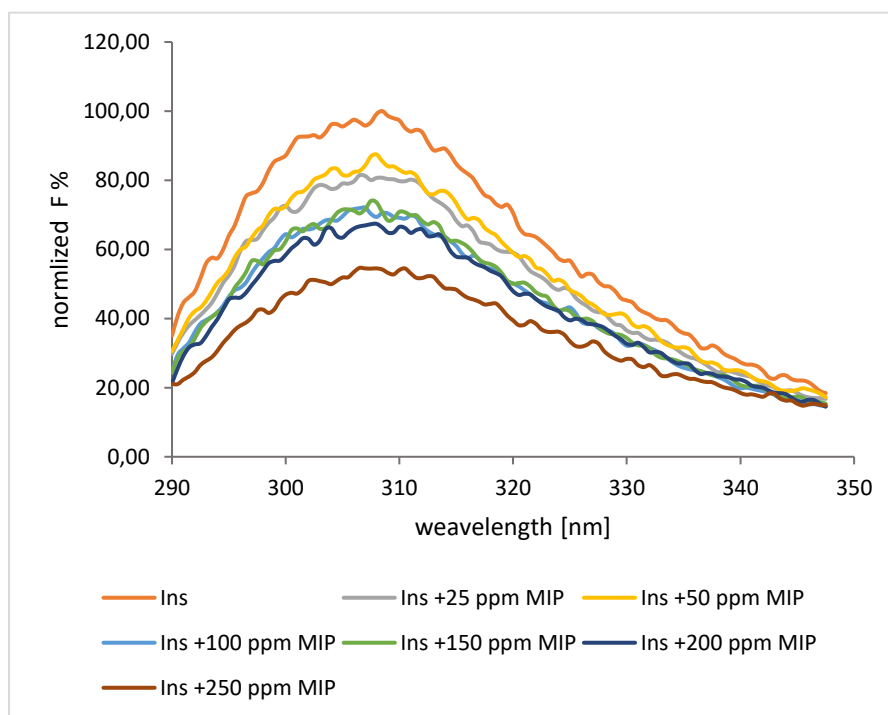


Figure 3. 13: Normalized fluorescence spectra of insulin (50 ppm) with increasing concentrations of HA nano-MIPs from batch EE05 (25–250 ppm).

The difference in binding efficiency between batches EE04 and EE05 cannot be explained by the monomer composition alone, since APM was not used in both formulations. A more likely reason is the technological nuances of the synthesis, for example: slight deviations in the dosing/mixing of reagents or the ambient temperature. As a result, the nano-MIPs of EE05, despite overall functionality and stability, show less pronounced binding compared to EE04.

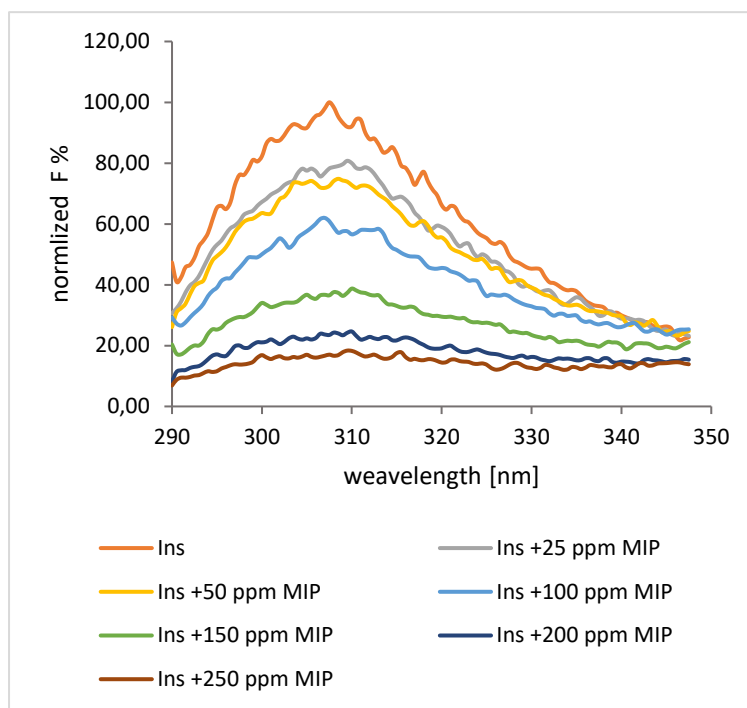


Figure 3. 14: Normalized fluorescence spectra of insulin (50 ppm) with increasing concentrations of HA nano-MIPs from batch EE06.

As shown in **Figure 3.14**, a pronounced and highly consistent quenching effect was observed, with fluorescence intensities decreasing progressively as the MIP concentration increases. At the highest concentration of 250 ppm, the signal at 307.5 nm was reduced by approximately 80-85%, indicating strong binding interactions and efficient recognition between the imprinted cavities and insulin molecules.

The spectral shape remained intact throughout the titration, with no detectable shifts in the emission maximum or spectral distortions. This suggests that the interaction did not affect the photophysical properties of insulin but instead resulted in classical static quenching due to complex formation or surface adsorption.

Compared to previous batches, EE06 demonstrated the strongest quenching efficiency, which may be attributed to a combination of factors: optimal particle size (as confirmed by DLS), a sharp and monodisperse size distribution, and well-formed binding sites with high accessibility. The binding profile was consistent with a high-affinity system approaching saturation, particularly in the concentration range of 150-250 ppm.

These results highlight EE06 as the most functionally responsive batch produced, exhibiting excellent colloidal properties, minimal aggregation, and superior quenching performance under the given conditions.

3.4.4 QCM Analysis

To further evaluate the binding properties and selective recognition of the synthesized nano-MIPs, QCM measurements were performed using high-affinity fractions of EE04, EE05, and EE06 batches. These analyses aimed to investigate the real-time interaction behavior with insulin under various experimental setups, including concentration series injections and competitive assays. The following sections describe the individual QCM results and observations for each batch.

Batch EE04

The QCM response obtained for the high-affinity fraction of batch EE04 is shown in **Figure 3.15**. Two channels were used during the measurement: Channel 1, functionalized with 6 mM insulin, and Channel 2, blocked with 100 mM ethanolamine. Only HA fractions at 500 ppm were tested.

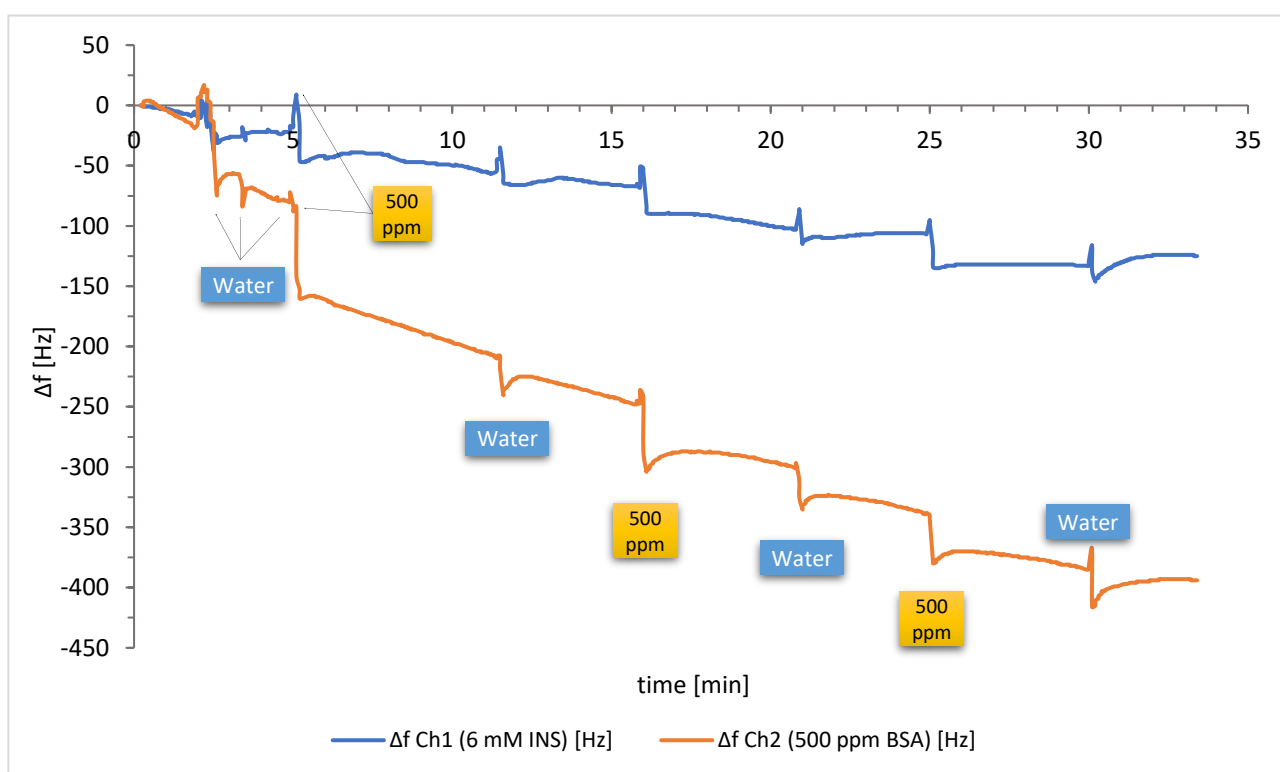


Figure 3. 15: QCM response to HA nano-MIPs (500 ppm) from batch EE04 injected onto an insulin-functionalized channel (blue) and an ethanolamine-blocked control channel (orange).

Five sequential injections of nano-MIPs were carried out, interleaved with rinses using MQ water to remove unbound or loosely adsorbed material from the sensor surface. Following each MQ water injection, only a partial recovery of the frequency signal is observed, suggesting that a significant portion of the MIPs remained bound even after washing. This behavior implies strong binding, although the specificity of the interaction was unexpectedly low.

Notably, the overall frequency shift on the ethanolamine-blocked reference channel (Channel 2) exceeded that of the insulin-functionalized surface (Channel 1), reaching ~ -400 Hz and ~ -100 Hz, respectively. This indicates predominant non-specific adsorption of nano-MIPs, likely related to

residual surface activity, incomplete blocking, or non-selective surface affinity. Conversely, the modest response on the insulin-coated channel may reflect suboptimal accessibility of the immobilized insulin or limited recognition efficiency by the MIP cavities.

Batch EE05

The experiment (**Figure 3.16**) included sample series from 50 to 500 ppm, applied on two channels: Channel 1, modified with 6 mM insulin, and Channel 2, serving as a reference, functionalized with 500 ppm bovine serum albumin (BSA).

Sequential injections of nano-MIPs at increasing concentrations were conducted, each followed by MQ water rinsing, with the exception of a procedural oversight during the first 200 ppm injection, where

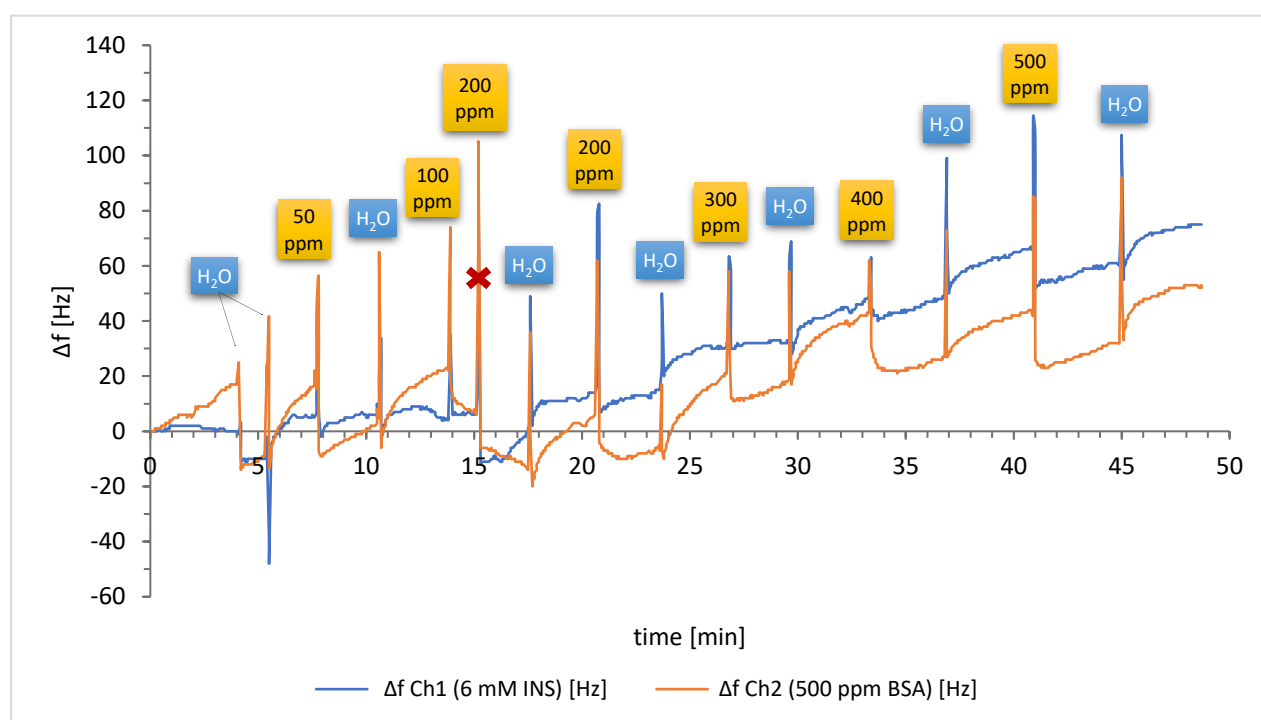


Figure 3. 16: QCM binding response of HA nano-MIPs (batch EE05) on insulin-functionalized (blue) and BSA-functionalized (orange) channels. Concentrations of nano-MIPs ranged from 50 to 500 ppm. MQ water rinses were applied between injections, except for the first 200 ppm point (marked by red cross).

rinsing was omitted. The signal recovery following this step confirmed that the surface was not irreversibly altered.

The insulin functionalized surface (Channel 1) displays measurable binding, though the absolute response remained moderate throughout the experiment. This suggests either limited affinity or restricted accessibility of the immobilized insulin.

One potential explanation for the reduced binding efficiency is the absence of a molecular spacer during surface functionalization. Since insulin is a relatively large molecule, steric hindrance or unfavorable orientation may have reduced the effective exposure of recognition binding site motifs to

the incoming MIPs. This hypothesis was later addressed in subsequent experiments (see EE06) by incorporating a 1-Butanethiol spacer into the QCM surface chemistry. Furthermore, an unusual upward drift in frequency is observed throughout the experiment. This behavior contradicts the typical frequency decrease expected from mass accumulation. While some non-classical responses may occur due to solvent effects or surface elasticity changes, in this case the most plausible explanation is a slight mechanical instability or improper sealing of the QCM cell, possibly leading to flow artifacts or leakage. As a result, although some degree of adsorption is evident, the experimental limitations preclude conclusive interpretation of specific binding in this batch.

Batch EE06

In the case of EE06, a series of QCM experiments were conducted to assess both the selective binding behavior of the nano-MIPs and their saturation behavior under several concentration series conditions, culminating in a competitive binding assay to evaluate specific insulin-MIP interactions in solution. To begin, the selectivity of the EE06 nano-MIPs was assessed using QCM sensors that had been pre-functionalized with either 6 mM insulin or 500 ppm BSA, covalently immobilized on the sensor surface via a cysteamine-glutaraldehyde coupling strategy. In this batch, 1-butanethiol was introduced as a molecular spacer during the surface functionalization process to reduce steric hindrance and improve accessibility of the binding sites, particularly for the relatively large insulin molecules. Onto these prepared surfaces, nano-MIPs were injected in increasing concentrations from 50 to 500 ppm. As shown in **Figure 3.17**, the insulin-coated surface exhibits a consistent decrease in resonance

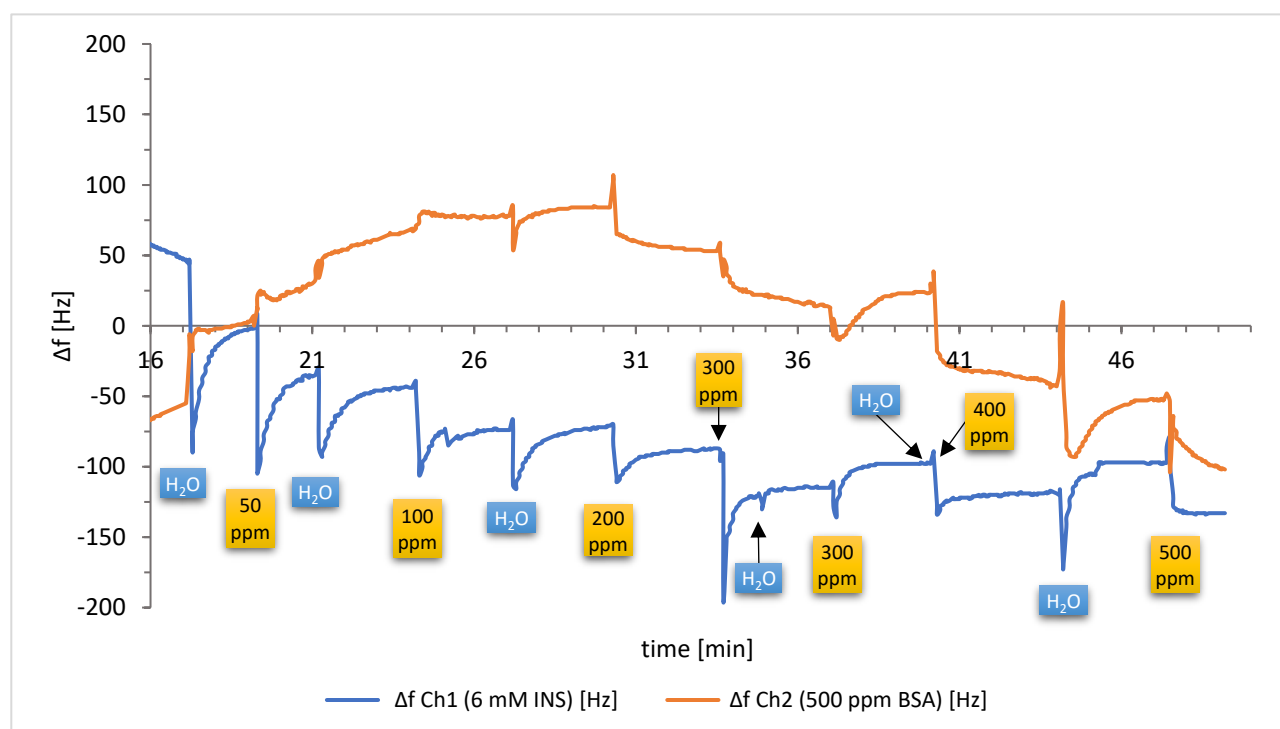


Figure 3. 17: QCM frequency response of EE06 nano-MIPs injected at increasing concentrations (50–500 ppm) onto insulin- and BSA-functionalized channels.

frequency upon successive injections of nano-MIPs, indicating progressive binding and accumulation of particles. In contrast, the BSA-coated channel demonstrated minimal or erratic responses, suggesting low-affinity, nonspecific interactions. This behavior supports the selectivity of the nano-MIPs towards insulin over the model protein.

The insulin-only trace (**Figure 3.18**) clearly illustrates a trend towards lower frequencies, typical of successful adsorption on a functionalized surface. Notably, the injection of 300 ppm nano-MIPs was accidentally performed without a prior water rinse, potentially leading to a denser or more disordered accumulation. After a subsequent rinse, a second 300 ppm injection was performed, which led the frequency to slightly increase, likely due to partial surface regeneration.

Following this, injections of 400 and 500 ppm nano-MIPs result in smaller responses, indicating that the surface was nearing saturation. Taken together, the inflection point appeared around 300-400 ppm, beyond which additional material did not lead to substantial binding signal increases.

It is important to highlight that only short intervals (approximately five minutes) were allowed between injections, which may have been insufficient for full stabilization of the sensor signal. This is visible in the non-horizontal plateaus between steps, especially at early concentrations, and may partially affect the accuracy of quantifying subtle response differences.

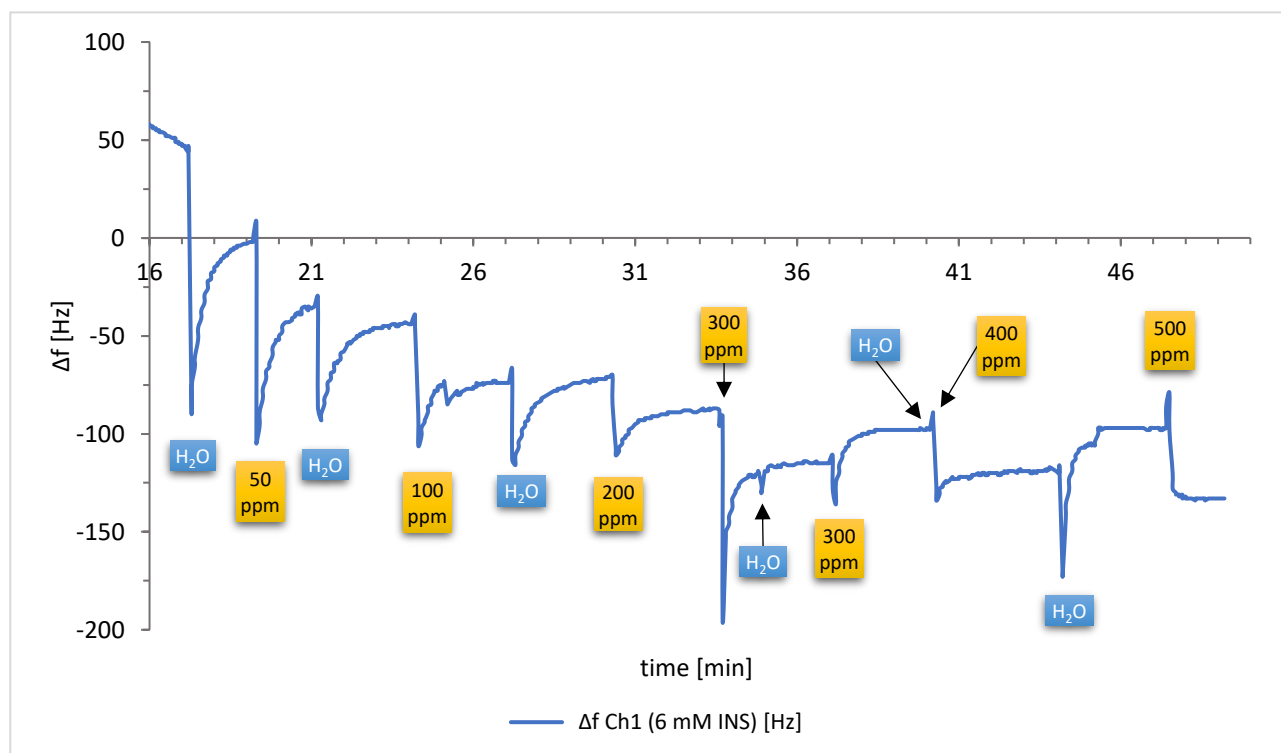


Figure 3. 18: Overlay of the insulin-coated channel only, showing the stepwise frequency shifts from each MIP injection. A saturation trend is visible from 300 ppm onwards.

To further examine the binding selectivity of EE06 nano-MIPs, both insulin- and BSA-functionalized QCM surfaces were exposed to a series of samples ranging from 50 to 500 ppm. As shown in **Figure 3.19**, a clear concentration-dependent response was observed in both channels, with a progressively decreasing resonance frequency (Δf) after each injection.

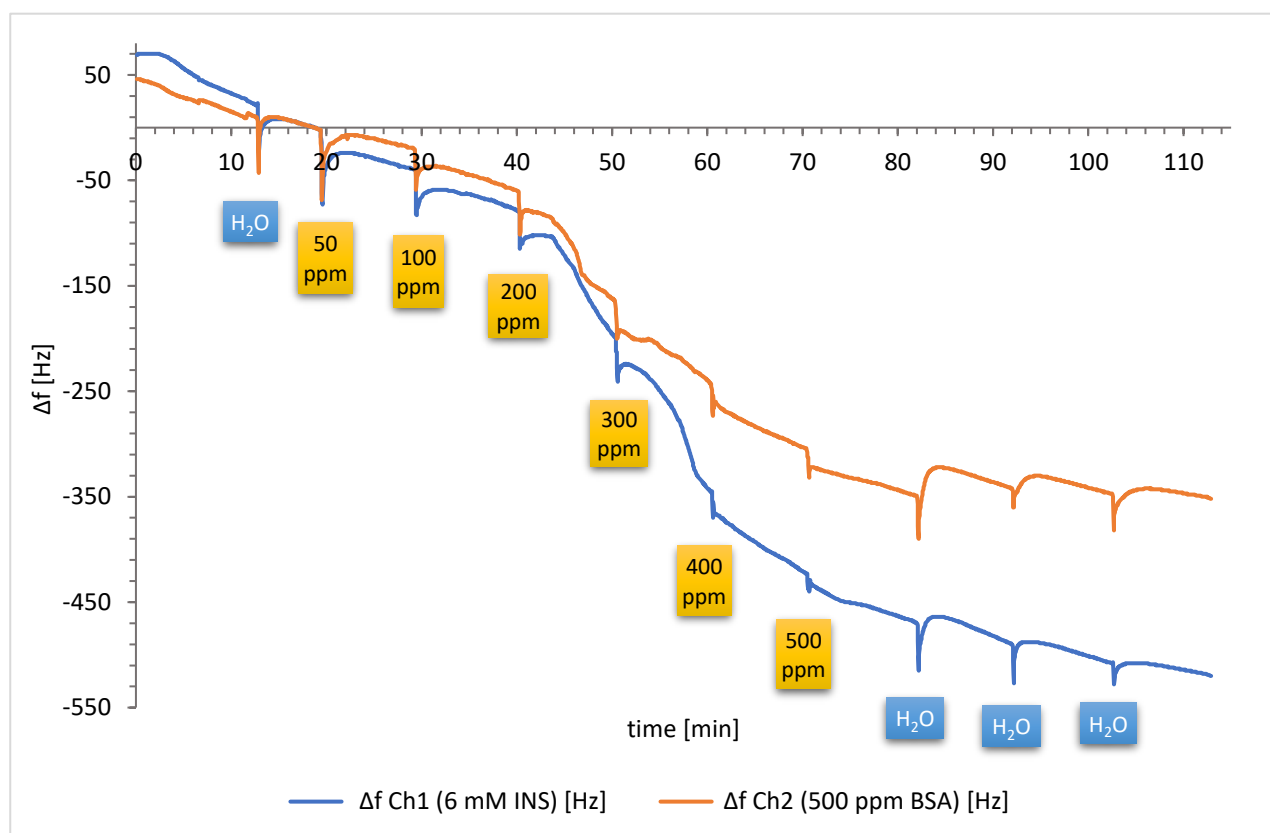


Figure 3. 19: QCM frequency responses upon injection of increasing concentrations of EE06 nano-MIPs (50–500 ppm) onto surfaces functionalized with 6 mM insulin (blue) and 500 ppm BSA (orange).

While a stronger and more distinct response was expected in the insulin-functionalized channel (Ch1, blue), a notable decrease in frequency was also observed in the BSA-functionalized channel (Ch2, orange), especially after the 300 ppm injection and onwards. However, the frequency shifts on the insulin channel are consistently larger across all concentrations, indicating a preference for insulin binding. For instance, at 500 ppm, the insulin-functionalized surface reaches a shift of approximately -420 Hz, compared to about -310 Hz on the BSA-functionalized side, giving a channel difference of roughly 110 Hz (all values are referenced to the stabilized baseline immediately before the first sample injection). This difference reflects the preferential binding of particles toward insulin and highlights their selective interaction under the applied conditions. Despite this clear preference, the BSA-functionalized channel still shows a noticeable response, indicating a certain degree of non-specific adsorption. Such behavior may stem from incomplete template removal, residual functional groups capable of electrostatic or hydrophobic interactions, or partial structural similarity between insulin and BSA binding motifs. These effects do not outweigh the overall selectivity but explain the measurable shifts on the reference channel.

For better visualization, the insulin-specific channel is presented separately (**Figure 3.20**), clearly highlighting the progressive and concentration-dependent frequency drop across the full measurement.

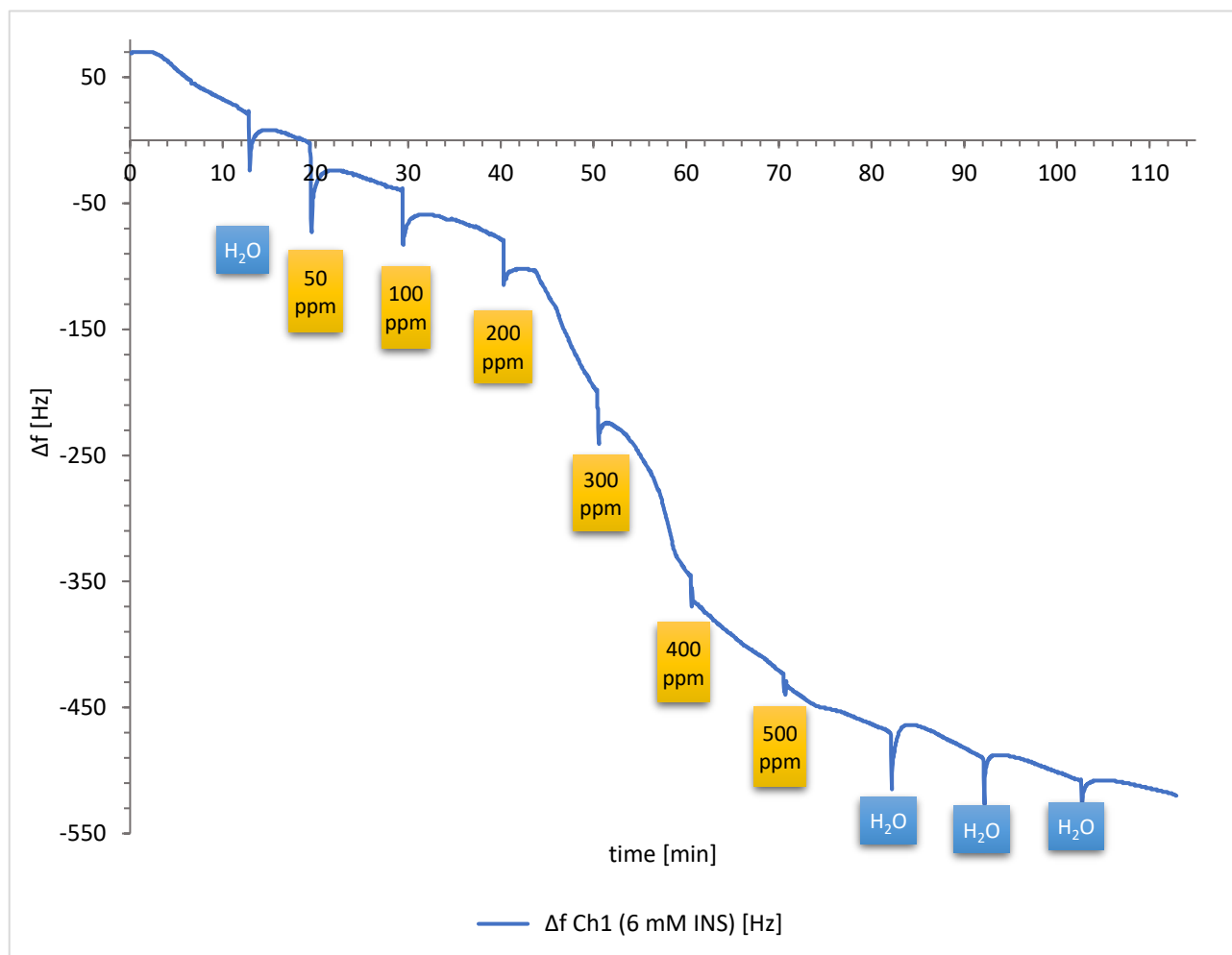


Figure 3. 20: Frequency response of the insulin-functionalized QCM channel upon sequential injection of EE06 nano-MIPs (50–500 ppm). The graph is extracted from the same measurement shown in Figure 3.19.

To define an appropriate concentration of EE06 nano-MIPs for use in the competitive binding assay, a preparatory QCM experiment (**Figure 3.21**) was conducted using a concentration series of nano-MIPs (50–300 ppm).

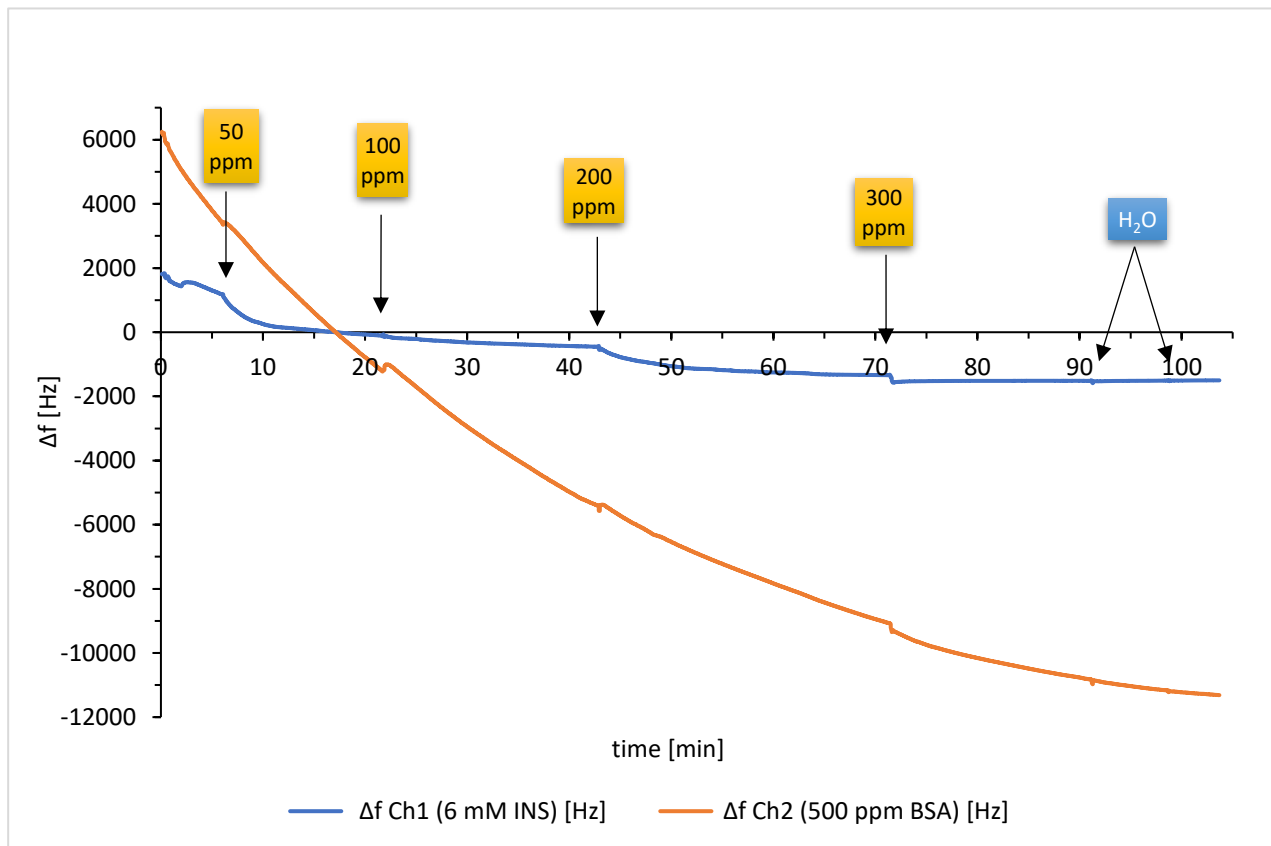


Figure 3. 21: QCM measurement showing the response of EE06 nano-MIPs (50–300 ppm) on an insulin-functionalized channel (blue) and a BSA-functionalized reference channel (orange).

As shown in **Figure 3.21**, MIP injections led to progressive decreases in frequency on both the insulin-functionalized (Ch1, blue) and BSA-functionalized (Ch2, orange) channels. The blue trace consistently exhibits more pronounced shifts, indicating stronger and more specific binding to insulin, while the BSA-functionalized channel showed a steady baseline drift with almost constant slope, suggesting that no actual binding occurred. The signal likely reflects instrumental instabilities rather than specific interactions, which makes a quantitative comparison with the insulin channel unreliable.

Beyond 200 ppm, the incremental frequency changes diminish significantly, particularly on the insulin-coated surface. This saturation trend is clearly illustrated in **Figure 3.22**, where only the insulin channel is displayed for improved clarity. The curve flattens from 200 ppm onward, suggesting that most available binding sites were occupied and that further increases in MIP concentration yielded marginal additional binding.

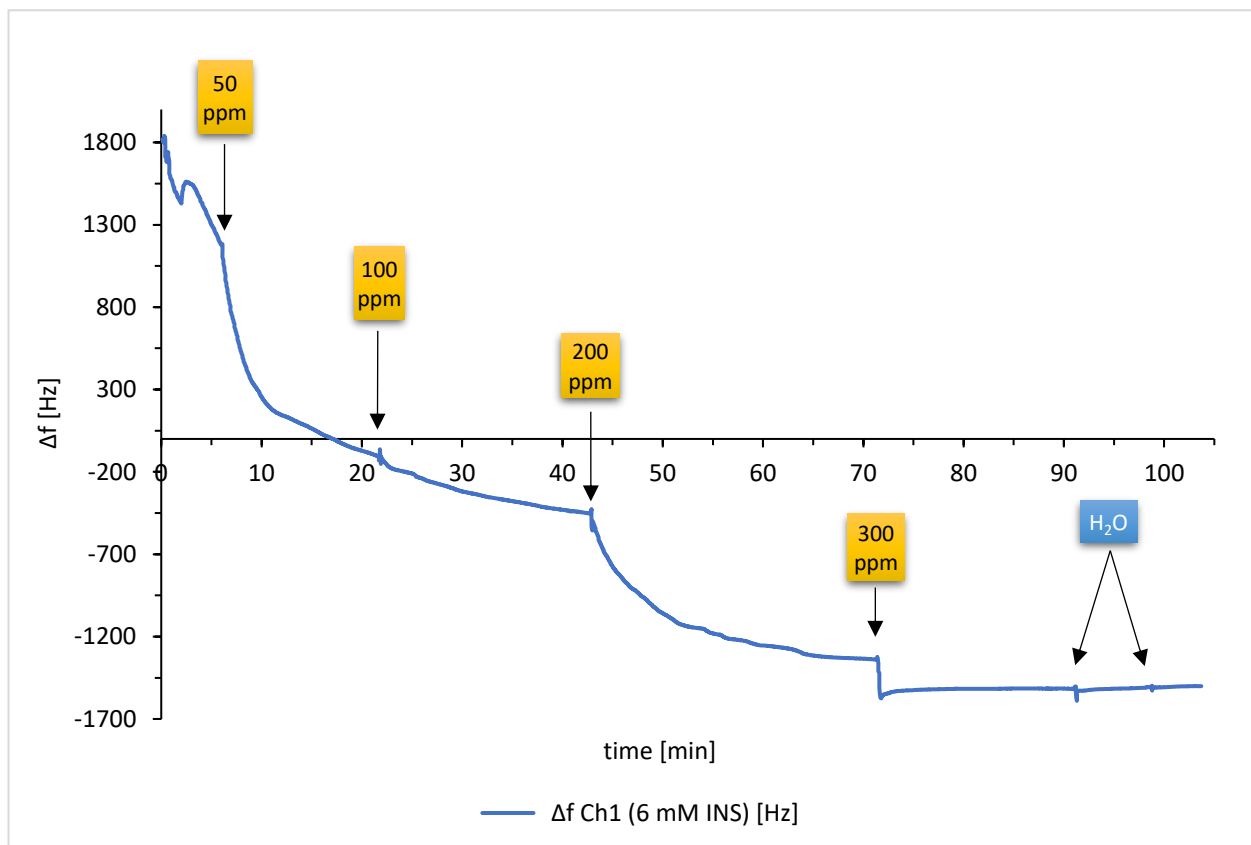


Figure 3. 22: Same data as in Figure 3.21, showing only the insulin-functionalized channel for clarity. The saturation point appears to be reached between 200 and 300 ppm, as the frequency shifts level off beyond this concentration.

Based on these results, 200 ppm was selected as the working MIP concentration for subsequent competitive experiments, ensuring sufficient surface occupancy while avoiding oversaturation.

Competitive Binding Assay with EE06 nano-MIPs

To further evaluate the binding performance of the EE06 nano-MIPs in solution, a QCM-based competitive assay was carried out using a fixed nano-MIP concentration of 200 ppm and a decreasing concentration series of insulin (20, 15, 10, 5, 0 ppm).

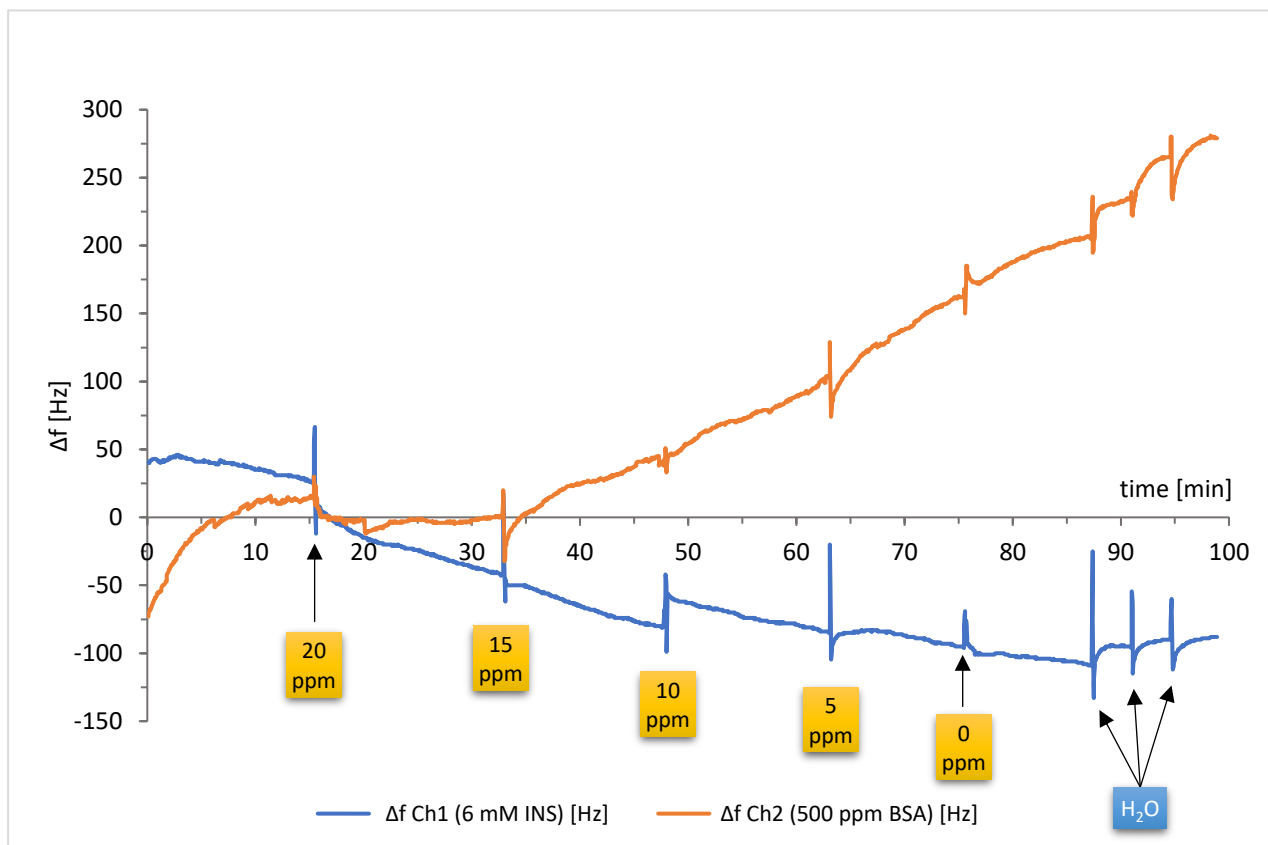


Figure 3. 23: QCM-based competitive binding assay using 200 ppm EE06 nano-MIPs pre-incubated with decreasing insulin concentrations (20 to 0 ppm). Blue trace: insulin-functionalized channel. Orange trace: BSA-functionalized channel.

As shown in **Figure 3.23**, only a marginal change in frequency shift is observed with decreasing insulin concentration, particularly on the insulin-functionalized channel (Ch1, blue). This suggests that the pre-saturation of MIP binding sites in solution may have been insufficient, or that the incubation time prior to injection was too short for efficient competitive binding.

For improved clarity, the insulin-functionalized channel is shown separately in **Figure 3.24**, highlighting the subtle trend in signal decrease.

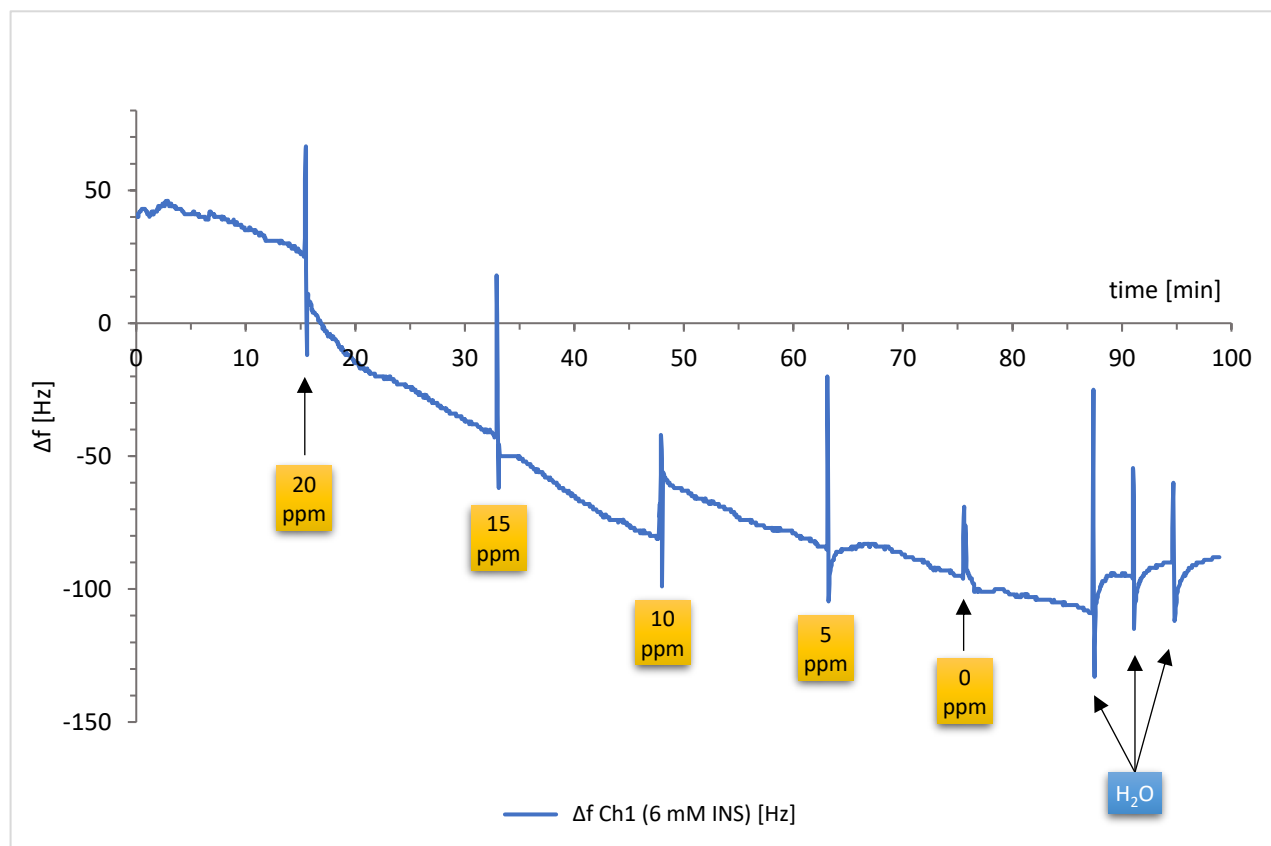


Figure 3. 24: Same dataset as in Figure 3.23, showing only the insulin-functionalized channel for clarity.

To further evaluate the binding performance of the EE06 nano-MIPs, a complementary competitive assay was conducted using a reversed insulin concentration series (200, 150, and 0 ppm). The goal was to verify whether a higher concentration of free insulin could fully saturate the binding sites on the nano-MIPs and thereby minimize their interaction with the immobilized insulin.

As shown in **Figure 3.25**, a clear decrease in frequency was observed in the insulin-functionalized channel (Ch1, blue) upon each injection of the nano-MIP mixture containing decreasing amounts of free insulin. The most pronounced frequency drop occurs at 0 ppm insulin, as expected, reflecting strong binding of the nano-MIPs to the surface-bound insulin. Notably, even at 200 ppm insulin, a significant response was still recorded, suggesting that the binding sites were not fully occupied by the free insulin molecules in solution. The reference channel (Ch2, orange), functionalized with BSA, showed minimal response across all concentrations, confirming the selectivity of the nano-MIPs even under challenging conditions.

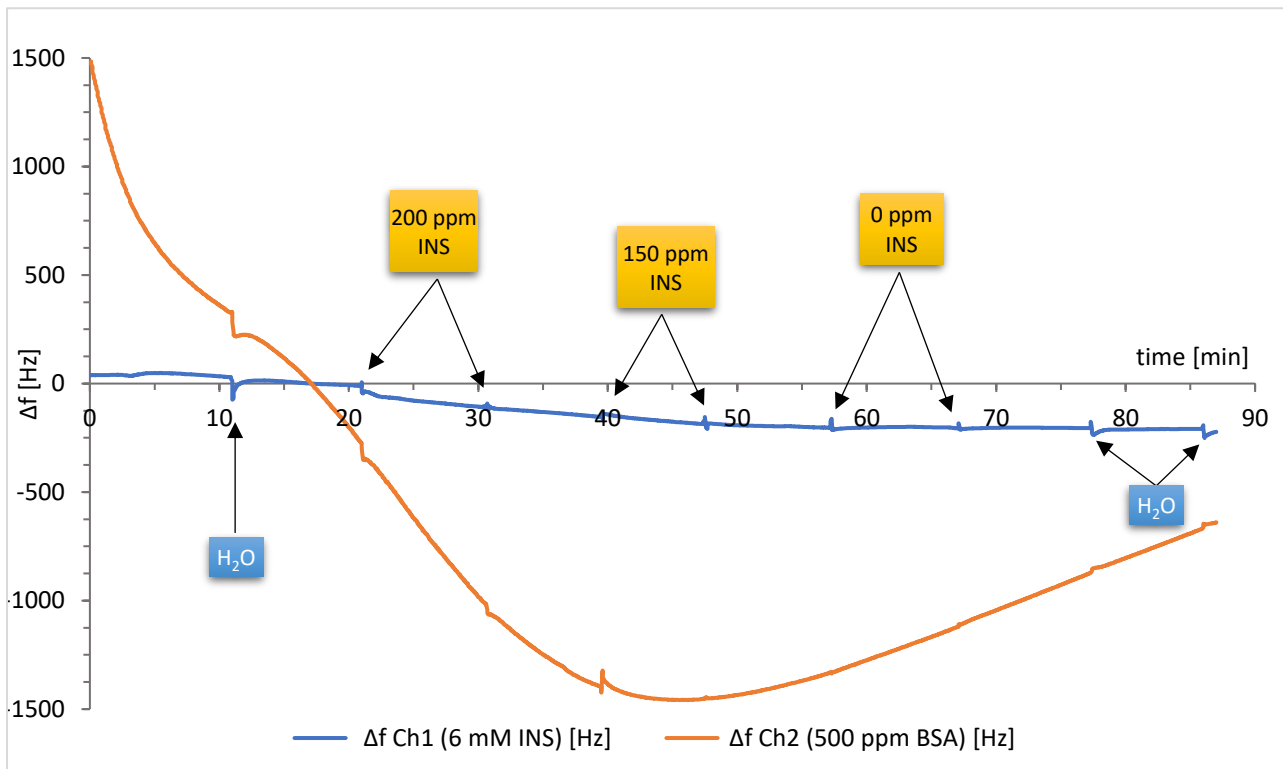


Figure 3. 25: Competitive assay with reversed insulin concentration series (200, 150, 0 ppm) using insulin- and BSA-functionalized channels (EE06).

For improved clarity, the insulin-functionalized channel is also shown separately in **Figure 3.26**, which illustrates the consistent trend of increasing frequency shift with decreasing insulin concentration, reaffirming the inverse relationship between free insulin in solution and surface binding.

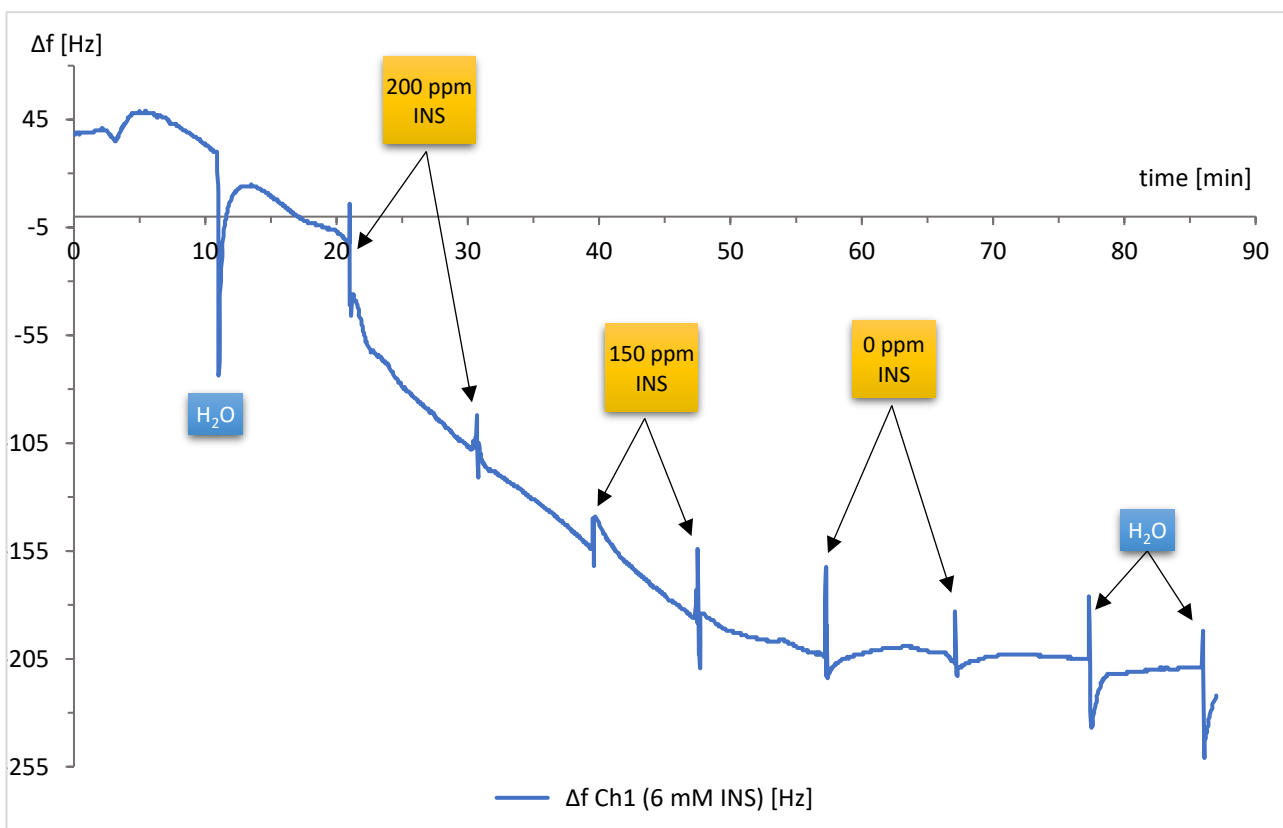


Figure 3. 26: Competitive assay shown for the insulin-functionalized channel only, highlighting the inverse correlation between insulin concentration in solution and QCM response.

This partial competition may stem from several factors. Based on the concentration range used in this assay, full saturation of the nano-MIPs is not observed: even at 150-200 ppm free insulin, the insulin: MIP ratio still remains in strong MIP excess, meaning that only a fraction of high-affinity sites becomes occupied at these concentrations.

Additional factors that may contribute include:

- insufficient incubation time for full equilibration,
- slight aging or degradation of the nano-MIPs affecting their binding performance, or
- a heterogeneous distribution of affinity among available binding sites i.e., only a fraction of high-affinity sites being saturated at the tested concentrations.

3.5 Challenges and considerations

Throughout the synthesis and characterization of insulin-specific nano-MIPs, several practical and conceptual challenges emerged, shaping both experimental design and interpretation. This section addresses these issues, providing technical context and reflective insights to guide future optimization of the workflow.

SEM characterization

In scanning electron microscopy, one of the main challenges was related to sample preparation. Given the relatively high average concentration of nano-MIPs used (typically around 500 ppm), the resulting dried layers were often too dense or aggregated, hindering proper visualization of individual particle morphology. While this particle concentration is acceptable for general overview, it reduced the ability to resolve discrete nanoscale features. Therefore, for future high-resolution SEM imaging, a lower concentration range (e.g., 100-200 ppm) should be considered, applied as a well-dispersed solution on the substrate and allowed to dry under controlled, gradual conditions to avoid clustering.

DLS and zeta potential

Dynamic light scattering and zeta potential analyses are highly time-sensitive. It was observed that postponing measurements even by a few days after synthesis can affect the accuracy and reproducibility of the size distribution and surface charge values, likely due to aggregation or degradation processes. For optimal results, DLS and zeta potential assessments should be performed immediately after synthesis and prior to any purification or prolonged storage steps.

Fluorescence assay

In fluorescence-based binding assays, homogeneity and timing are critical. The physical mixing of nano-MIPs and insulin must be thorough, as even small inconsistencies in dispersion could lead to variable binding efficiency and optical readouts. It is presumed that sedimentation of nano-MIPs over time might reduce their availability for binding if the assay is not executed promptly. Ensuring gentle agitation or vortexing immediately before the measurement and minimizing time gaps between mixing and analysis is therefore essential.

QCM experiments

Quartz crystal microbalance measurements presented the most intricate set of technical challenges:

1. **Device stability and sealing:** The QCM cell must be assembled with precise alignment and strong sealing, especially around the silicone ring and fluidic connectors. In some cases, improper sealing led to slow leakage or pressure fluctuations, affecting baseline stability and introducing noise into the signal. These effects may not be immediately visible during injection but can accumulate over time.
2. **Baseline stabilization:** Allowing for sufficient stabilization time before the start of measurement is essential. It was found that baseline drift or fluctuations were prominent when the recording began too soon after device initialization. For improved reproducibility, a pre-conditioning period of at least 20-30 minutes is recommended before baseline acquisition.
3. **Injection timing and incubation:** Several experiments, particularly competitive binding assays, were affected by insufficient incubation times between injections. MIP-analyte interactions require adequate contact time for equilibrium binding. Injecting successive samples too rapidly (e.g., after only 5-10 minutes) may lead to incomplete responses or underestimation of binding capacity. Later experiments benefited from longer incubation periods (~25-30 minutes), highlighting the importance of kinetic equilibrium.
4. **Surface saturation and unexpected responses:** In some datasets, such as **Figures 2.22** and **2.24**, saturation of the QCM surface by MIPs was observed beyond 200 ppm. However, even with increased insulin concentrations up to 200 ppm in the competitive binding solution, signal shifts continued to occur, indicating that not all binding sites were saturated. Possible explanations include insufficient incubation time, partial degradation or aging of the MIPs, or slow rebinding kinetics under flow conditions.
5. **Signal artifacts and injection peaks:** High, sharp frequency spikes at the moment of sample injection were regularly observed. These artifacts were attributed to mechanical disturbances

caused by manual pipetting into narrow microchannels, introducing sudden pressure changes. Although these peaks do not reflect molecular binding, they complicated the interpretation of the actual start of response. Future setups might benefit from automated injection systems to minimize such disturbances.

6. **Channel mismatch and baseline misalignment:** Some measurements revealed overlapping resonance frequencies between the two channels. When both reference and active channels oscillate at nearly identical frequencies (e.g., 9.940 MHz and 9.942 MHz), distinguishing their independent signals becomes difficult. Ideally, the two channels should differ by at least ~ 0.01 MHz to enable effective differential comparison. Additionally, baseline shifts in later runs occasionally moved the curves upward rather than downward, suggesting either unstable device behavior or subtle leakage, possibly from the improperly seated chamber.

RAFT polymerization and synthesis issues

The synthesis phase also presented critical considerations. In particular, it became evident that UV exposure must be minimized. Merely shielding the reaction vessel with aluminum foil proved insufficient. The most successful batch (EE06) was synthesized after sunset under near-total darkness, suggesting that stray UV or ambient light during polymerization can impair polymer quality. Maintaining inert atmosphere throughout the reaction, including during monomer addition and mixing is also crucial. In several cases, reagents were old, which could have led to stoichiometric imbalances. Using freshly prepared and fully dissolved solutions under nitrogen or argon would improve reproducibility.

Reflections

Despite these challenges, the experimental strategy proved effective for identifying and analyzing insulin-specific binding behavior using nano-MIPs. The systematic design of experiments from fluorescence to QCM allowed for cross-validation of selectivity and sensitivity. However, it became evident that procedural consistency, timing, and physical setup quality are major limiting factors when working at nanoscale resolution with real-time biosensing techniques.

Addressing these issues in future work will not only improve signal-to-noise ratios but also enable better kinetic modeling and interpretation of binding mechanisms. A more automated, standardized pipeline from synthesis to measurement would greatly enhance both reliability and throughput of nano-MIP-based biosensor development.

4. Conclusion and outlook

In this study, a series of insulin-specific nano-MIPs were synthesized and systematically characterized using a combination of techniques including fluorescence quenching, DLS and zeta potential measurements, SEM imaging, and QCM analysis. The aim was to optimize a solid-phase synthesis protocol for nano-MIPs tailored for insulin recognition, and to evaluate their physicochemical properties and binding performance.

Solid-phase synthesis protocols with and without the addition of APM as a functional monomer were compared. The absence of APM in later batches (EE04-EE06) leads to notable differences in binding behaviour and particle characteristics. Fluorescence quenching assays revealed varying degrees of analyte recognition across different batches, with EE04 and EE05 showing more pronounced quenching effects, while EE06 was selected for further in-depth analysis, including QCM experiments and competitive binding assays.

Size and charge measurements (DLS and zeta potential) confirmed the nanoscale range of the particles, with observed variations in distribution and stability. Importantly, SEM analysis underscored morphological differences between high and low affinity fractions, with high affinity samples exhibiting more distinct and less aggregated spherical structures.

The QCM studies provided real-time insights into the dynamic binding behaviour of nano-MIPs on functionalized gold surfaces. Selectivity tests demonstrated a distinguishable frequency response for insulin compared to BSA, confirming partial specificity. However, the competitive assays revealed limitations in pre-saturation and binding saturation, suggesting incomplete interaction under the applied experimental conditions. Potential explanations include insufficient incubation time, suboptimal particle stability due to aging, or limited availability of accessible binding sites.

Despite these challenges, the obtained results demonstrate the feasibility of producing insulin-recognizing nano-MIPs via a reproducible and adaptable solid-phase synthesis strategy. The experimental workflow - encompassing binding affinity separation, concentration adjustment protocols, and standardized measurement techniques can serve as a solid foundation for future optimization and integration into biosensing applications.

4.1 Key findings, limitations, future work

This work has identified several key findings:

- Solid-phase synthesis using non-APM protocols yields nano-MIPs with selective binding properties, albeit with batch-dependent variability.
- Fluorescence quenching and QCM results are sensitive to particle stability and sample preparation timing, highlighting the need for rapid post-synthesis analysis.
- Competitive assays show no clear trend across insulin concentration series, indicating the need for improved pre-binding strategies or increased incubation time.

Limitations include potential aggregation affecting SEM and DLS results, challenges in QCM device sealing, and variability in injection technique during QCM assays.

Future work could focus on:

- Exploring alternative spacers and surface chemistries to enhance MIP orientation and accessibility of binding sites.
- Developing a fully quantitative QCM calibration protocol with real-time kinetics modelling.
- Integrating freshly synthesized nano-MIPs into microfluidic platforms for more controlled and reproducible analyte delivery.

Abstract

Das molekulare Prägen hat sich als ein zentraler Ansatz zur Entwicklung synthetischer Erkennungssysteme etabliert, die eine hochselektive und stabile Bindung an Zielanalyten ermöglichen. Besonders vielversprechend sind in diesem Zusammenhang molekular geprägte Polymer-Nanopartikel (nano-MIPs), die aufgrund ihrer hohen Stabilität, Reproduzierbarkeit und Funktionalität unter unterschiedlichen physikochemischen Bedingungen eine attraktive Alternative zu Antikörpern darstellen. Dennoch bestehen weiterhin Herausforderungen hinsichtlich der Reproduzierbarkeit, Partikelgrößenkontrolle und Bindungsspezifität für den Einsatz in biosensorischen Anwendungen.

Ziel dieser Arbeit war die Entwicklung und umfassende Charakterisierung von insulin-spezifischen nano-MIPs, die mittels Festphasenimprinting und RAFT-Polymerisation in Dimethylformamid (DMF) synthetisiert wurden. Frühere Ansätze auf Basis der radikalischen Polymerisation in wässrigem Medium führten zu heterogenen und schlecht definierten Partikeln, was zur Umstellung auf ein RAFT-basiertes Protokoll im organischen Lösungsmittel führte. Dieses wurde hinsichtlich Partikelqualität, Stabilität und Affinität optimiert.

Die resultierenden nano-MIPs wurden mittels dynamischer Lichtstreuung (DLS), Zeta-Potenzial-Messungen, Rasterelektronenmikroskopie (REM) und Fluoreszenzspektroskopie charakterisiert. Die DLS-Analyse zeigte eine enge Größenverteilung mit einem durchschnittlichen Durchmesser von ca. 30 nm und einer Polydispersität unter 0.2. Zeta-Potenzial-Werte um ca. -30 mV deuteten auf eine gute kolloidale Stabilität hin. Die REM-Bilder bestätigten eine sphärische Morphologie mit homogener Oberflächenstruktur.

Fluoreszenz-Bindungsexperimente belegten die spezifische Interaktion mit Insulin: Bei konstanter Insulinkonzentration (50 ppm) und steigender nano-MIP-Konzentration (0–250 ppm) wurde eine progressive Fluoreszenzlöschung beobachtet, mit einem Sättigungsbereich zwischen 200 und 250 ppm.

Ein zentraler Bestandteil der Studie war der Einsatz der Quarzkristallmikrowaage (QCM), um sowohl direkte als auch kompetitive Bindung zu untersuchen. Die Goldoberflächen der Sensoren wurden mit Cysteamin und 1-Butanthiol modifiziert und anschließend mit Glutardialdehyd aktiviert. Danach wurden entweder Insulin oder bovines Serumalbumin (BSA) als Kontrollprotein aufgetragen. Bei Injektion eines nano-MIP-Konzentrationsserien (50–500 ppm) zeigte sich ein signifikant höheres Signal bei Insulin-gebundenen Oberflächen im Vergleich zu BSA, was die Selektivität der nano-MIPs bestätigte.

Für die kompetitiven QCM-Experimente wurden dieselben mit Insulin funktionalisierten Sensoren verwendet. Es wurden vorgemischte Proben injiziert, die eine konstante nano-MIP-Konzentration von 200 ppm enthielten, während die Insulinkonzentration schrittweise von 0 bis 200 ppm erhöht wurde.

Trotz zunehmender Insulinkonzentration konnte keine deutliche Hemmung der Bindung festgestellt werden, was möglicherweise auf eine unzureichende Inkubationszeit oder eine bereits teilweise Bindung im Lösungsgemisch zurückzuführen ist.

Insgesamt präsentiert diese Arbeit einen systematischen Ansatz zur Synthese und funktionellen Analyse von insulin-spezifischen nano-MIPs. Die Ergebnisse belegen ihre Selektivität in QCM-Messungen und unterstreichen ihr Potenzial für den Einsatz in biosensorischen Plattformen, etwa zur Diagnose von Diabetes oder in anderen Anwendungen, die eine zuverlässige molekulare Erkennung erfordern.

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Hinweis zur sprachlichen Unterstützung

Im Rahmen dieser Arbeit wurden für die sprachliche Umsetzung und den Übersetzungsprozess unterstützende Tools eingesetzt, insbesondere ChatGPT (OpenAI), DeepL und Google Translate. Die Nutzung dieser Werkzeuge diente ausschließlich der sprachlichen Unterstützung; sämtliche wissenschaftliche Inhalte, Ergebnisse und Schlussfolgerungen wurden von der Autorin selbstständig erarbeitet.