BUDAPEST UNIVERSITY OF TECHNOLOGY AND ECONOMICS
FACULTY OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY
GEORGE OLÁH DOCTORAL SCHOOL

Development of luminescent probe and nanoparticle-based analytical tools for the selective recognition and quantitation of biomolecules

Thesis booklet

Author: Ahmed Marwa Aly Ahmed Aly

Supervisor: Dr. Viola Horvath

Department of Inorganic and Analytical Chemistry

MTA-BME Computation Driven Chemistry Research Group

2022

1 Introduction and background

Biomolecules are chemical compounds present in living organisms. They are ranging from small molecules like primary metabolites and natural products to large molecules like proteins and complex carbohydrates (*Figure 1).* They play an important role in biological and biochemical processes. Because they are fundamental building blocks, their presence and proper concentrations are critical for the structure and proper functioning of living cells. Any variation in the concentration of specific biomolecules may indicate the onset of a disease. Therefore, e.g. any change in the lysozyme level in human urine or serum can be used for the diagnosis of health problems. Elevated urine and/or serum concentrations are observed in rheumatoid arthritis, renal dysfunction, sarcoidosis and extremely high serum (5–230 mg/mL) and urinary (up to 1600 mg/L) concentrations are observed in monocytic and myelomonocytic leukemia[1]. Nucleotides, such as adenosine diphosphate (ADP), guanosine monophosphate (GMP), and adenosine monophosphate (AMP) play essential roles in metabolism, bioenergetics, synthesis of nucleic acids and other metabolic processes[2]. N-acetyl amino acids are also important small biomolecules. N-acetyl histamine (NAC-him) and N-acetyl histidine (NAC-his) are frequently used ligands to mimic the binding properties of the metal binding side chain imidazole residues of proteins. N-acetyl aspartic acid (NAC-Asp) is present in very high concentrations in mammalian brain. It is used as an index to determine changes in brain metabolites and as a marker for neuronal death and neuronal injury[3].

Therefore, accurate measurement of the concentration of specific biomolecules are critical to monitor the health of living organisms.

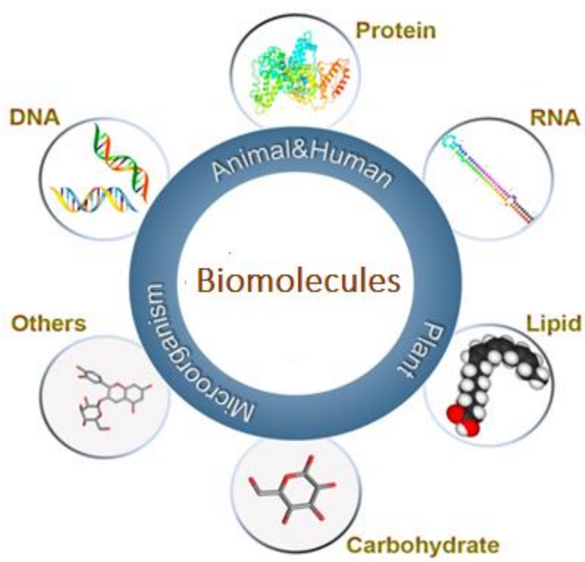

Figure 1: Different types of biomolecules present in living organisms[4]

[1] Pruzanski W, *Am. J. Med. Sci.*, vol. 258, no. 6. pp. 405–415, 1969.

[2] Hess and Greenberg, *Nutr. Clin. Pract.*, vol. 27, no. 2, pp. 281–294, 2012.

[3] Birken DL, *Neurosci. Biobehav. Rev.*, vol. 13, no. 1, pp. 23–31, 1989.

[4] Jia M et al., *Nanomaterials*, vol. 8, no. 9, pp. 730, 2018.

State-of the art analytical techniques for the quantitation of small and macromolecular biomolecules are based on chromatography-mass spectrometry and ligand-binding assays[5,6,7] While the former requires highly sophisticated instruments, the latter approach uses fragile antibodies of biological origin, which are costly to develop and have short shelf-life.

Currently, there is a lot of interest in developing selective capturing and simple and fast sensing methods for the determination of biomolecules *in vitro* or *in vivo*.

Sensor molecules are capable of selectively recognizing the target analyte, thereby producing a simultaneous change of a physico-chemical signal. By far the most frequently applied signaling scheme is fluorescence, owing to its high sensitivity and selectivity.

Molecular sensing and recognition systems for nucleotides and amino acids has received significant interest recently because of their biological importance[8,9]. Luminescent sensor molecules are successful tools to monitor in vitro or in vivo biologically relevant species due to their high sensitivity and simplicity. In the last decades lanthanide metal complexes, especially the complexes of Eu^{3+} and Tb^{3+} have attracted much attention, due to their strong luminescence emission, long luminescence lifetime, narrow emission band and large Stokes' shift[10]. The intra-configuration 4f–4f transitions in lanthanide ions are parity forbidden, consequently their absorption and emission spectra show weak intensity. If the Ln^{3+} ion forms a complex with an organic ligand, the ligand absorbs and transfers energy efficiently to the metal ion and consequently increases its luminescence intensity. The ligand selected has, most commonly, aromatic or heterocyclic moiety with a conjugated π-system that can absorb the excitation energy and donate it to the Ln^{3+} ion. The most common ligands used are coumarin and its derivatives[11,12]. **In my work I developed a new luminescent probe containing a new derivative of coumarin, named 8-allyl-2-oxo-2H-chromene-3-carbaldehyde (AOCC) and used it to quantify small biomolecules. Also, I extensively studied the nature of the binding process. (Paper I)**

Polymer nanoparticles (PNPs) are also extensively investigated as capturing devices/sorbents to bind biomolecules with high affinity and selectivity, due to their extremely high surface area and highly variable chemical structure. Moreover, by using a stimuli-sensitive polymer as the backbone, they can swell and collapse in response to environmental changes, such as temperature, pH, ionic strength and light[13]. Shea's group synthesized thermally responsive poly(N-isopropylacrylamide-*co*-N-tert-butylacrylamide-*co*-acrylic acid) PNPs to selectively capture and release lysozyme upon temperature change[14]. The monomer composition was carefully optimized because a delicate balance between the hydrophobic N-tert-butylacrylamide

---

[5] Baskal S et al., *Amino Acids*, vol. 57, no. 4, pp. 615–641, 2021.
[6] Tang N and Bhat VB, *J Biomol Tech*, vol. 23, p.S55., 2012.
[7] Brouwer J et al., *Clin. Chim. Acta*, vol. 142, no. 1, pp. 21–30, 1984.
[8] Agafontsev AM et al., *Chem. A Eur. J.*, vol. 25, no. 11, pp. 2684–2694, 2019.
[9] Staszak K et al., *Coord. Chem. Rev.*, vol. 397, pp. 76–90, 2019.
[10] Alam AM et al. , *Bull. Korean Chem. Soc.*, vol. 33, 2012.
[11] Georgieva I et al., *J. Inorg. Biochem.*, vol. 135, pp. 100–112, 2014.
[12] Gudasi KB , *Chem. Pharm. Bull.*, vol. 53, no. 9, pp. 1077–1082, 2005.
[13] Agrawal G and Agrawal R, *Polymers.*, vol. 10, no. 4, 2018.
[14] Yoshimatsu K et al., *Angew. Chemie*, vol. 124, no. 10, pp. 2455–2458, 2012.

(TBAm) and the negatively charged acrylic acid (AAc) was crucial to achieve high selectivity. During thermal polymerization at 60 ^{0}C with persulfate initiator, they used a surfactant to control the size of the PNPs. Piletsky's and Haupt's groups prepared protein-imprinted PNPs using similar polymer compositions via the so-called "solid-phase synthesis method"[15,16]. For this purpose, they had to modify the polymerization conditions because the protein template would have been denatured at 60 ^{0}C and in the presence of a detergent. So they used tetramethylethylenediamine (TEMED) that works as an activator of the persulfate initiator at room temperature and surfactant-free polymerization. However, no one investigated the consequence of this modification. Moreover, a disadvantage of the both the non-imprinted and imprinted PNPs produced by these groups, is that the swollen or collapsed hydrogel nanoparticles are very difficult to handle and separate due to their small size and similar density to water.

In my work, I addressed the questions concerning the initiator system (Paper II) and explored different ways to improve the usability of these PNPs, (i) by building a magnetic core into them, thereby simplifying their separation (Paper III) and (ii) by introducing a novel separation-free method to quantitate protein-nanoparticle binding (unpublished).

I have compared two redox-initiator systems, namely persulfate/TEMED and persulfate/$NaHSO_3$ that allow room temperature polymerization, to thermal initiation using persulfate with or without surfactant for the preparation of multifunctional lysozyme-selective PNPs. I have studied, how these different initiator systems influence the microstructure and the lysozyme binding properties of the PNPs (Paper II).

To facilitate the handling and separation of the PNPs, I have prepared a thin layer of the thermoresponsive lysozyme-selective hydrogel on magnetic core nanoparticles (Lys-PMNP) using controlled polymerization. I have elaborated a sample pretreatment method to selectively extract lysozyme from human urine samples. The analytical applicability of Lys-PMNPs for the clinical assessment of lysozymuria was demonstrated by bioanalytical method validation. (Paper III).

I have also addressed the inconvenient separability of PNPs by developing a novel separation-free method to quantitate the protein-polymer nanoparticle interaction. The technique is based on fluorescence anisotropy measurement using a long-lifetime Ru-complex. The protein is labeled with this fluorophore, the long lifetime of which enables the depolarization of the emitted light by the rotation of the protein. However, when the protein is bound to the much larger polymer nanoparticle, the rotation slows down considerably, and the emitted light remains polarized. The method can be used for the quantitation of proteins in a competitive ligand binding assay format and can also give thermodynamic and kinetic information on the nanoparticle-protein binding event. (unpublished result.)

2 Experimental

2.1 Preparation of polymer nanoparticles

Based on the procedure reported by Lyon and Debord[17], I have prepared thermoresponsive PNPs from N-isopropylacrylamide (NIPAm), acrylic acid (AAc) and tert-butyl

[15] Guerreiro A et al., *Adv. Healthc. Mater.*, vol. 3, no. 9, pp. 1426–1429, 2014.
[16] Ambrosini S et al., *Chem. Commun.*, vol. 49, no. 60, pp. 6746–6748, 2013.
[17] Debord JD and Lyon LA, *Langmuir*, vol. 19, no. 18, pp. 7662–7664, 2003.

acrylamide (TBAm) monomers with N,N'-methylenebis(acrylamide) (BIS) crosslinker. *Table 1* shows the feed monomer composition and the other synthesis conditions of the nanoparticles. A total monomer concentration of 65 mM was used. The polymerization was carried out in a thermostated water bath for three hours under argon atmosphere using 2.6 mM ammonium persulfate (APS) and the same concentration of N,N,N'N'tetramethylenediamine (TEMED) or sodium bisulfite (SBS) initiator.

Table 1: Polymerization conditions used during the synthesis of the nanoparticles

Sample name	Monomer composition [mol%]				Initiator system (molar ratio of the components)	Temperature [°C]
	AAc	TBAm	NIPAm	BIS		
PNP1*	5	40	53	2	APS	60°C
PNP2	5	40	53	2	APS	60°C
PNP3	5	40	53	2	APS:SBS (1:1)	40°C
PNP5	5	40	53	2	APS:TEMED (1:1)	40°C

**** PNP1 prepared in the presence of sodium dodecyl sulfate (SDS) surfactant.***

2.2 Preparation of lysozyme-selective core-shell magnetic nanoparticles

I have prepared nanosized Fe_3O_4 (MNPs) using solvothermal method[18] then a silica shell was deposited onto the MNPs using a sol-gel method[19] (MNPs@SiO_2). I have modified the silica with amino groups by using 3-aminopropyltrimethoxysilane (APTMOS)[20] (MNPs@SiO_2-NH_2). A RAFT agent, 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid was immobilized to the MNPs@SiO_2-NH_2 nanoparticles by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride to allow controlled polymer growth from their surface[21] (*Figure 2*). A thin layer of polymer was coated onto the MNPs@SiO_2@RAFT particles (Lys-PMNP) by controlled radical polymerization using the monomer composition and polymerization condition of PNP3 (see section 2.1).

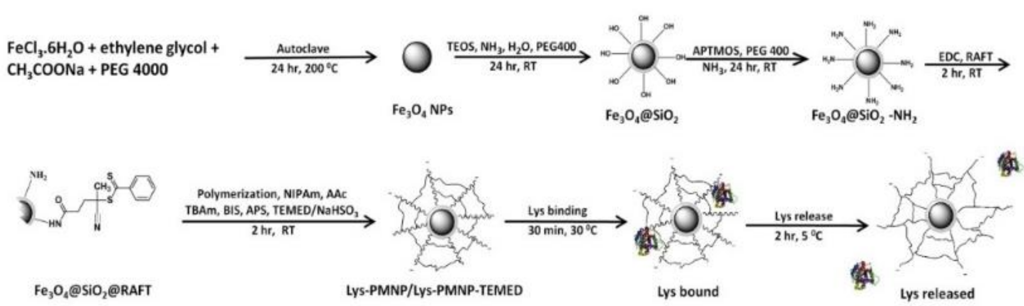

Figure 2: A schematic representation of Lys-PMNP preparation

---

[18] Deng H et al., *Angew. Chemie*, vol. 44, no. 18, pp. 2842–2845, 2005.
[19] Stöber W and Fink A, *Colloid Interface Sci.*, vol. 26, no. 1, pp. 62–69, 1968.
[20] Balogh-Weiser D et al., *Nanomaterials*, vol. 10, no. 12, p. 2329, 2020.
[21] Erdőssy J et al., *Anal. Methods*, vol. 9, no. 31, pp. 4496–4503, 2017.

3 Results and discussion

3.1 A new luminescent bio-probe, Europium (III) -8-Allyl-2-oxo-2H-chromene-3-carbaldehyde (AOCC) for sensing of biomolecules

I have developed a new Eu^{3+}-(AOCC)$_3$ bio-probe by combining Eu^{3+}metal ion with a novel coumarin derivative ligand (AOCC)$_3$ and used it as fast, simple sensor for the quantitation of N-acetyl amino acids, nucleobases, nucleotides and DNA.

First, I confirmed the formation of the Eu^{3+}-(AOCC)$_3$ complex by measurement of the excitation and emission spectra of 1μM Eu^{3+}-(AOCC)$_3$-probe in ethanol at λ_{em} = 616 nm and λ_{ex} = 350nm (*Figure 3a)*. The strong emission intensity of Eu^{3+} ion at 616 nm in the presence of the ligand (AOCC) suggests that AOCC is a good sensitizer of the Eu^{3+} ion.

I have studied the interaction of the Eu^{3+}-(AOCC)$_3$ complex with various biomolecules, Eu^{3+}-(AOCC)$_3$ by adding different concentrations of biomolecules and following the luminescence intensity changes. *Figure 3b* shows the luminescence spectra of 1 μM Eu^{3+}–(AOCC)$_3$ complex after the addition of different concentrations of NAC-Asp between 0.1–1 μM in ethanol. It was found that the biomolecules have a remarkable effect on the fluorescence intensity of the Eu^{3+}-(AOCC)$_3$ complex by quenching it.

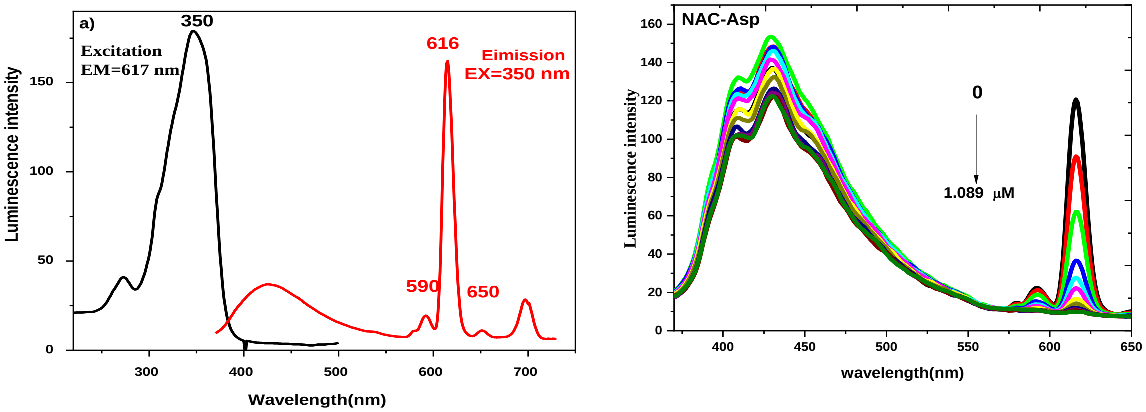

Figure 3: Excitation and emission spectra of 1μM Eu^{3+}-(AOCC)$_3$-probe in ethanol (a); Emission spectra of the Eu^{3+}-(AOCC)$_3$-probe (1 μM) with different concentrations of NAC-Asp (b) in ethanol at RT

I developed a rapid and simple, time-resolved (gated) luminescence-based method for the determination of these biomolecules in microtiterplate using the long-lived Eu^{3+}-(AOCC)$_3$-probe. To verify the mechanism of quenching, the luminescence data has been analysed at 4 different temperatures (298 K, 303 K, 308 K and 313 K) in the concentration range from ≈0.1 to 1 μM, using the Stern–Volmer equation ($F_0/F = 1+K_{sv}$ c), where K_{sv} is the Stern–Volmer constant, F and F_0 are the luminescence intensities of the Eu-(AOCC)$_3$ complex in the presence and absence of the biomolecule, respectively and c is the concentration of the biomolecule.

The experimental results indicated that the probable quenching mechanism of the Eu^{3+}-(AOCC)$_3$-probe fluorescence is static quenching, because lower K_{sv} values were obtained when the temperature increased.

The corresponding binding constants were determined at different temperatures by using the Stern-Volmer equation. It was established that one biomolecule forms a complex with the Eu^{3+}-(AOCC)$_3$ complex.

The nature of the interaction between the Eu^{3+}-(AOCC)$_3$-probe and the biomolecules has been investigated by determining the thermodynamic parameters using the Vant- Hoff equation ($\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$, where R is the gas constant, T is the temperature, and K is the binding constant). In case of NAC-Asp, NAC-Lys, NAC-his, NAC-him, adenine, cytidine, inosine, 5′-IMP, 5′-CMP, 5′- ATP and 5′-ADP ΔH and ΔS are negative, indicating Vander Waals force or hydrogen bond formation. In the case of, 5′-GMP ΔH has a small negative value, while ΔS has a positive value, suggesting an electrostatic interaction. The large value for the entropy changes also suggests that the binding process is mostly entropy-driven.

3.2 Optimization of the initiator system of thermoresponsive poly(NIPAm-*co*-AAc-*co*-TBAm) microgels designed for molecular imprinting of lysozyme

Mild polymerization conditions in molecular imprinting of the fragile proteins are imperative. In order, to imprint poly(NIPAm-*co*-AAc-*co*-TBAm) nanoparticles (PNPs), instead of thermal initiation at 60 °C, close to room temperature polymerization is needed. Surfactants can also denature the protein, therefore their presence has to be avoided. I have set out to investigate what effects are encountered in PNPs, when these polymerization conditions are changed. I have synthesized PNPs using two redox initiator systems, APS with TEMED and APS with SBS around room temperature and, for comparison, with the thermal activation of APS in the presence or without surfactant. SEM micrographs of PNP1, PNP2, PNP3 and PNP5 are shown in *Figure 4 a-d.* Particle sizes obtained with SEM together with the polydispersity indices (U) are listed in *Table 2*.

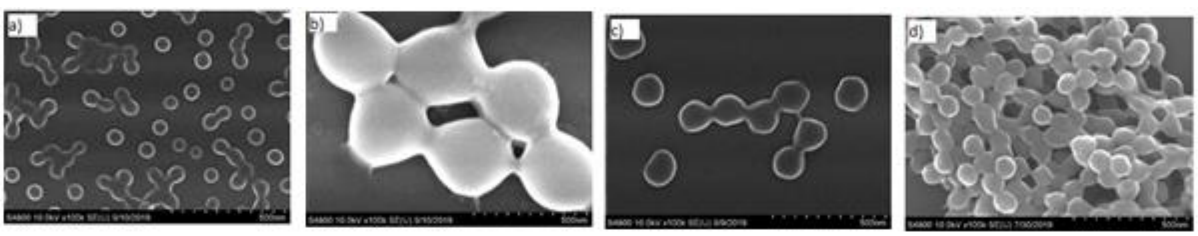

Figure. 4: SEM images of PNPs: a) PNP1, prepared at 60°C using SDS; b) PNP2, prepared at 60°C without SDS; c) PNP3, prepared at 40°C using SBS and d) PNP5, prepared at 40°C with TEMED.

Figure 4 shows that thermal and APS/SBS initiation resulted in highly monodisperse colloidally stable nanoparticle suspensions. On contrary, the APS/TEMED system afforded monodisperse but extensively aggregated nanoparticles.

Table 2: Particle size and polydispersity index of the nanoparticles

Sample	Dry particle diameter [nm]	U
PNP1	70.2±9.4	1.057
PNP2	374±22	1.011
PNP3	152±14	1.026
PNP5	93.4±6.7	1.015

During polymerization I have followed the consumption of each monomer by HPLC. Conversion of each monomer was calculated and plotted as a function of the total monomer conversion to visualize their relative incorporation rate (*Figure 5*). In these plots if a monomer has a more positive curvature than the other it indicates that it is converted faster throughout the reaction. The relative incorporation rates of the different monomers showed unique characteristics in case of the APS/TEMED initiator compared to the other systems. While using the latter ones the conversion rate of AAc was comparable to the other monomers, using TEMED the relative incorporation rate of AAc was extremely slow.

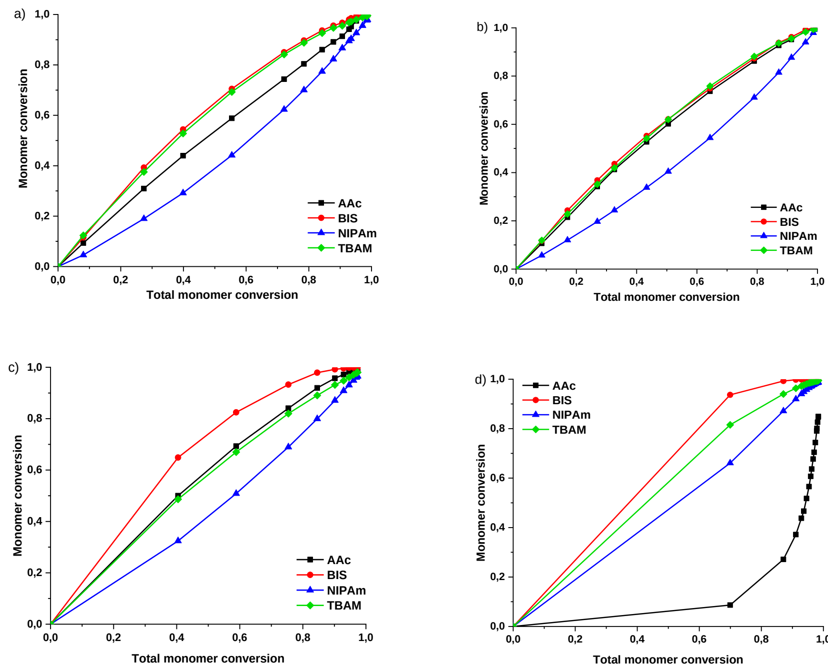

Figure 5: Conversion of the different monomers as a function of the total monomer conversion a) PNP1 prepared at 60ºC using SDS; b) PNP2 prepared at 60ºC; c) PNP3 prepared at 40ºC with the APS/SBS initiator system; d) PNP5 prepared at 40ºC with the APS/TEMED initiator system

Looking more closely into the polymerization rates of the individual monomers, it turned out, that TEMED selectively enhanced the polymerization rates of all other monomers, but AAc. Applying higher feed concentrations of AAc, or lowering the TEMED concentration, the relative polymerization rates of the monomers became more similar. I attributed this to the acid base reaction between TEMED and AAc, i.e. the protonation of TEMED and the deprotonation of AAc. It is known from literature that the negatively charged acrylate monomer has inherently lower reactivity than acrylic acid[22,23]and that the protonated TEMED is less active[24]. The differences observed in the dissimilar relative incorporation rates of acrylic acid between the TEMED catalyzed and the thermally or SBS initiated polymerization systems led to very diverse particle microstructures. While the other systems afforded almost homogeneous incorporation of AAc throughout the particle, the TEMED activated reaction rendered AAc to accumulate almost exclusively in the outer shell of the nanoparticles.

I have measured the binding of lysozyme to the nanoparticles using different PNP concentrations. Lysozyme binding capacity of the nanoparticles was estimated from the data obtained and can be seen in *Table 3*.

---

[22] Cutié SS et al., *Polym. Sci. Part B Polym. Phys.*, vol. 35, no. 13, pp. 2029–2047, 1997.
[23] Kabanov VA et al., *Polym. Sci. Polym. Symp.*, vol. 42, no. 1, pp. 173–183, 1973.
[24] Caglio S and Righetti PG, *Electrophoresis*, vol. 14, no. 1, pp. 554–558, 1993.

Table 3: Estimated lysozyme binding capacity of the nanoparticles

Sample	Bound lysozyme per unit surface area [μg/m^2]
PNP1	1700
PNP2	1200
PNP3	1000
PNP5	250

As can be concluded from Table 3 PNP1 (SDS at 60°C), PNP2 (at 60°C) and PNP3 (SBS at 40°C) binds lysozyme in a similar order of magnitude. This suggests that they show similar affinity to the protein. Polymer PNP5 (TEMED at 40°C) binds almost an order of magnitude less protein showing a significantly decreased affinity. It is presumed that the microstructural inhomogeneity in these particles disrupted the delicate balance of hydrophobic and charged areas on the particle surface leading to a decreased binding of lysozyme.

Due to the inherent basic properties of TEMED, its application in the polymerization of multifunctional nanoparticles containing acidic monomers requires much attention and careful optimization. SBS, a similar redox activator of APS offers a viable alternative when the synthesis temperature should be kept low, because it affords nanoparticles with similar microstructure and protein binding properties as the thermally initiated polymerization.

3.3 Temperature-responsive magnetic nanoparticles for the bioanalysis of lysozyme in urine samples.

In order, to advance the use of protein-selective PNPs for the sample-preparation of lysozyme from urine samples, I coated the crosslinked poly(NIPAm-co-AAc-co-TBAm) hydrogel onto magnetic nanoparticles, therefore their separation became very fast and easy.

During the step-by-step synthesis of Lys-PMNPs XRD, FT-IR and zeta-potential measurements were done to confirm the formation of each layer. The morphology of the Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@SiO_2-NH_2 and Lys-PMNP particles was characterized by TEM and SEM measurements. The polymer shell thickness was roughly estimated from the TG measurements.

I have studied different parameters which influenced the lysozyme binding property of Lys-PMNPs, like incubation time, pH, buffer ionic strength, presence of NaCl, temperature of binding or release and finally the selectivity towards different proteins.

Selectivity of the Lys-PMNPs towards various proteins was assessed by comparing the amount of nanoparticle bound protein in buffered solutions of lysozyme, avidin, horseradish peroxidase C (HRP C), bovine serum albumin (BSA), albumin from chicken egg, human serum albumin (HSA), γ-globulin and cytochrome C (cyt C) at RT. The results are shown in *Figure 6.*

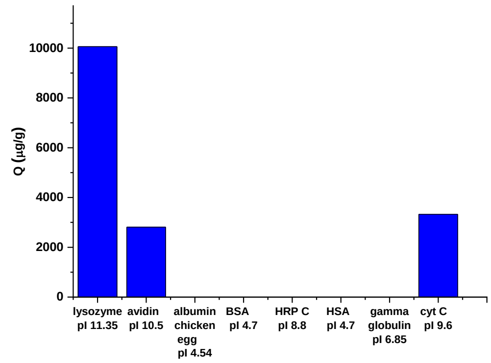

Figure 6: Selectivity of Lys-PMNPs towards other proteins at RT in buffer systems. (1.6 mg/mL Lys-PMNP was incubated with 50 µg/mL protein solution in 50 mM PB, pH 7)

We found the Lys-PMNPs show no binding with BSA, chicken egg albumin, HSA and γ-globulin having acidic or neutral, and HRP C with weakly basic isoelectric point. Moderate binding with Cyt C and avidin, with more basic pI values was observed.

We have also investigated the selectivity of the Lys-PMNPs in the presence of high concentrations of the two clinically relevant proteins, HSA and γ-globulin, because these might interfere with lysozyme in different diseases accompanied by proteinuria. I applied these proteins together with lysozyme in the highest concentration of their abnormal range, but no interference was found. This indicates that the Lys-PMNPs show extremely high selectivity, which potentiates their use in clinical analysis of lysozyme in the presence of other highly abundant proteins.

I was also motivated to elaborate a bioanalytical method using these core-shell magnetic nanoparticles (Lys-PMNPs) as selective sorbent. For binding of lysozyme I diluted the urine sample 10 times with 10 mM phosphate buffer (PB) (pH 8.4) and incubated the sample with Lys-PMNP for 30 min at 30^{0}C, then I collected the nanoparticles with a magnet. After washing the particles with water I resuspended them in 50 mM PB (pH 7.4) and left the samples at 5^{0}C for 2 hours to release the bound lysozyme. The released lysozyme was quantitated by HPLC.

Finally, I validated the method according to bioanalytical method validation guidelines[25], thereby fostering the application of protein-selective MNPs from research stage to routine analysis. Selectivity was assessed by injecting blank and spiked diluted urine samples with and without the sample cleanup procedure and lysozyme standard solutions. The chromatograms can be seen in *Figure 7*.

[25] European Medicines Agency, "Guideline on bioanalytical method validation," 2011.

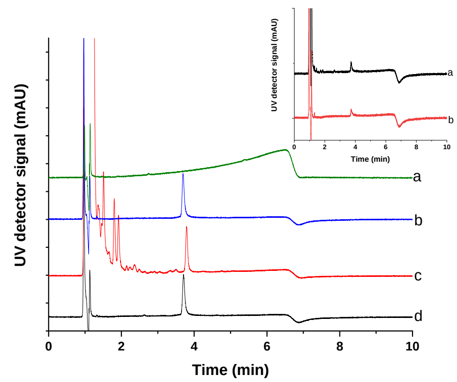

Figure 7: Chromatograms of a ten times diluted blank urine sample after pretreatment (a), a 10 µg/ml lysozyme standard (b), a 10 µg/ml diluted urine sample without sample pretreatment (c) and a pretreated 10 µg/ml diluted urine sample (d). (The retention time of lysozyme is 3.7 minute). The inset shows the chromatogram of a 2.5 µg/ml diluted urine sample after pretreatment (LLOQ sample) (a) and that of a 2.5 µg/ml lysozyme standard (b).

By comparing the chromatograms of the spiked urine sample before and after the sample cleanup process (*Figure 7c and d*) the high efficiency and selectivity of the Lys-PMNPs becomes evident. The lower limit of quantification (LLOQ) was 25 µg/ml. The method was linear in the 2.5-200 µg/ml concentration range (corresponding to 25-2000 µg/ml urine concentration). Intra- and inter-day precision values ranged from 2.24 to 8.20 % and 1.08 to 5.04 %, respectively. Intra-day accuracies were between 89.9 and 117.6 %, while inter-day accuracies were in the 88.8 to 111.0 % range. The average recovery was 94.1±8.1 %. Analysis of unknown urine samples in comparison with a well-established reference method (enzymatic assay of lysozyme) revealed very good correlation between the results, indicating that the new nanoparticle-based method has high potential in the diagnosis of lysozymuria.

3.4 A novel technique for the investigation of protein binding to polymer nanoparticles based on fluorescence anisotropy measurement using long-lifetime fluorophores

I demonstrated a new concept by studying the interaction of lysozyme labeled with bis (2,2'-bipyridine)-4'-methyl-4carboxypyridine-ruthenium N-succinimidyl ester bis (hexafluoro-phosphate) (RuL-Su) complex and the poly(NIPAm-co-AAc-co-TBAm) nanoparticles (PNPs) by using steady state fluorescence anisotropy measurement.

The label, RuL-Su have a long life time ~400 ns, and its steady state anisotropy is close to zero (unmeasurably low). When the RuL-Su was conjugated to lysozyme the rotational correlation time was increasing (the rotation of the protein conjugate become slower, than that of the free fluorophore) due to the large protein molecule, but owing to the long lifetime of the fluorophore, the anisotropy did not increase too much (0.0045). On contrary, when the RuL-Lys bound to the nanoparticle, an enormous increase in size arose, followed by significant rise in the anisotropy value (0.12). Based on this, if the labeled protein is mixed with PNPs the binding to the nanoparticles can be quantitated by measuring the anisotropy.

The binding isotherm was measured and an adapted Hill model was fitted on the data points with a good fit. The isotherm parameters are shown in *Table 5*.

Table 5: Parameters, and R^2 of the adapted Hill fitting

n_{max} (mol/g)	K_D (M^{-1})	n	R^2
$1.93 \cdot 10^{-3}$	$1.21 \cdot 10^{-7}$	0.435	0.9973

The $1.21 \cdot 10^{-7}$ M^{-1} value of $K_{D,}$ the dissociation constant, indicates quite strong interaction between the protein and the nanoparticles.

From n_{max}, the maximum binding capacity of the PNPs and the molar concentration of the nanoparticles we could estimate the average number of proteins that are bound to the surface of a PNP. Based on this, the maximum number of lysozyme molecules that can bind to a PNP is 154.000.

The value of n, being smaller than 1 (0.435), indicates anti-cooperative binding. This is a realistic scenario, since lysozyme is a highly positively charged protein at pH 7.4, with a pI value of 11.35, therefore repulsive forces are hindering the adhesion of a next protein close to an already adsorbed one.

In a competitive binding assay, we have applied the long-lifetime fluorophore-labeled proteins to quantitate lysozyme concentrations with the PNPs as "plastic antibodies. In this experiment the amount of the labeled protein was chosen so that ≈80% of it was bound to PNPs initially. Then I added increasing concentrations of unlabeled lysozyme and measured the steady state anisotropy. The characteristic S-shaped curve of competitive immunoassays was obtained, when the anisotropy values were plotted as a function of the logarithm of unlabeled lysozyme concentration, indicating that it was successfully competing for the PNP binding sites with the labeled protein. The IC50 value i.e. the analyte concentration where half of the labeled protein is bound was evaluated from the curve as being $3.2 \cdot 10^{-4}$ M.

The proposed method is fast, sensitive, requires simple instrumentation and does not require separation of the nanoparticle-bound and free-protein for quantitation. Even fast binding kinetics can be measured real-time.

4 Theses

1. I have developed a bio-probe, based on a new complex of Europium (III) with 8-Allyl-2-oxo-2H-chromene-3-carbaldehyde (AOCC) ligand for sensing small biomolecules (N-acetyl amino acids, nucleobases and nucleotides) and DNA with time-gated luminescence. The method is based on the fluorescence quenching of the Eu^{3+}-(AOCC) complex by the bound biomolecules. The stoichiometry of the biomolecule- Eu^{3+}-(AOCC) complex is 1:3, the mechanism of quenching is static. The affinity binding constants and the type of secondary interactions were determined. [Paper I]
2. I have demonstrated, for the first time, that the use of the tetramethylethylenediamine/ ammonium persulfate initiator system in the synthesis of poly(NIPAm-co-TBAm-co-AAc) nanoparticles leads to the uneven distribution of acrylic acid through the nanoparticle interior, which in turn negatively effects its affinity towards lysozyme. I have explained this behaviour with the acid base interaction of tetramethylethylenediamine and acrylic acid. [Paper II; Poster presentation 1, 2 and Oral presentation 4]
3. I prepared novel, poly(NIPAm-co-TBAm-co-AAc) hydrogel-coated thermoresponsive magnetic nanoparticles for the bioanalysis of lysozyme in urine. I have developed and optimized a protocol to selectively extract lysozyme from human urine samples. For the first time, I demonstrated the propriety of selective polymer nanoparticles in routine clinical analysis of proteins from real biological samples, by bioanalytical method validation. [Paper III; Poster presentation 3]
4. I have introduced fluorescence anisotropy measurement, as a novel separation-free method to quantitate the interaction of proteins with nanoparticles using a long-lifetime ruthenium-complex for labeling. I have demonstrated its use by measuring the binding of

lysozyme on poly(NIPAm-co-TBAm-co-AAc) nanoparticles. This new principle allows the quantitation of proteins by selective polymer nanoparticles, as antibody substitutes, in a competitive ligand-binding assay. [unpublished results]

5 Practical applicability of the results

The novel fluorescent Ln^{+3}-complex can be used to selectively measure essential biomolecules based on their fluorescence quenching. The time-resolved fluorescence measurement in the microtiter plate format is a very simple and cheap high-throughput assay, there is no need for harmful solvents and sophisticated chromatographic instrumentation. Also, the Eu^{3+}-(AOCC) complex is equipped with a polymerizable group, so this reporter molecule could be incorporated into MIPs in the future, thereby achieving even higher selectivity for the target biomolecule and also signalling of the binding event. (Paper I)

The use of SBS with APS initiator instead of TEMED for room temperature polymerization of multifunctional PNPs containing acidic monomers, leads to more homogeneous incorporation of the different monomers. This positively affects the particle surface, i.e. its monomer composition is more similar to the feed monomer composition, which is optimized to bind a specific protein. Therefore, the protein binding affinity of the PNPs is not deteriorated when room temperature polymerization is used. (Paper II)

I have demonstrated by method validation that the lysozyme-selective, thermoresponsive polymer-coated magnetic nanoparticles can perform well in routine bioanalytical applications as extraction sorbents. By varying the monomer composition or by molecular imprinting, their selectivity can be tuned for other proteins, too. They offer distinct advantages over current bioanalytical sample preparation techniques, such as high adsorption capacity, magnetic separability and the thermocontrollability of the binding event. These protein-selective polymer-coated magnetic nanoparticles can be used as cheap and robust alternatives of the fragile antibodies in binding assays, as well. (Paper III)

Nowadays, the extensive use of nanoparticles in biological systems either *in-vivo* or *in-vitro*, requires the full understanding of how different proteins interact with the nanoparticles. The fluorescence anisotropy measurement using long-lifetime fluorescent labels can be generally used to characterize the protein-nanoparticle interactions. The technique is separation-free, sensitive, fast and low-cost, and allows to gain not only thermodynamic but kinetic information on the protein-nanoparticle binding event. It requires small amounts of protein for the measurement, but as a drawback it requires the use of labeled protein. It can be generally applied for the quantitation of proteins using selective polymer nanoparticles in a competitive assay. (unpublished result)

6 Publications

[I] H. A. Azab, G. M. Khairy, N. Abd El-Ghany, and **M. A. Ahmed**, “A new luminescent bio-probe of Europium(III)-complex for sensing some biomolecules and CT-DNA,” *Photochem. Photobiol. A Chem.*, vol. 374, pp. 1–9, 2019. IF: 3.306, I:2, FI:2.

[II] **M. A. Ahmed**, J. Erdőssy, and V. Horváth, “The role of the initiator system in the synthesis of acidic multifunctional nanoparticles designed for molecular imprinting of proteins,” *Period. Polytech. Chem. Eng*, vol. 65(1), pp. 28–41, 2021. IF: 1.744, I:1, FI:-

[III] **M. A. Ahmed**, J. Erdőssy, and V. Horvath, "Temperature-Responsive Magnetic Nanoparticles for Bioanalysis of Lysozyme in Urine Samples," *Nanomaterials*, vol.11 (11), pp. 3015, 2021. IF: 5.719, I:1, FI:1.

Oral presentations and posters:

1. M. A. Ahmed, J. Erdőssy, and V. Horvath, "Optimization of the polymerization conditions of thermoresponsive poly(NIPAm-co-AAc-co-TBAm) microgels for the molecular imprinting of lysozyme" Graduate Student Symposium on Molecular Imprinting – GSS MIP 2019, August 28th-30th 2019 Berlin, Germany
   Poster presentation (BEST POSTER AWARD)

2. M. A. Ahmed, J. Erdőssy, and V. Horvath, "Optimization of the polymerization conditions of thermoresponsive poly(NIPAm-co-AAc-co-TBAm) microgels for the molecular imprinting of lysozyme" 23th Oláh György Doctoral School Conference, September 2019, Budapest, Hungary
   Poster presentation

3. M. A. Ahmed, J. Erdőssy, and V. Horvath, "Preparation of surface-modified core-shell magnetic nanoparticles for the imprinting of proteins" 30th Oláh György Doctoral School Conference, January 2019, Budapest, Hungary
   Poster presentation

4. M. A. Ahmed, and V. Horvath, "The Role of the Initiator System in the Synthesis of Acidic Multifunctional Nanoparticles Designed for Molecular Imprinting of Proteins" Online conference for Young Researchers' International Conference on Chemistry and Chemical Engineering (YRICCCE III), June 4-5, 2021, Romania.
   Oral presentation