THESES OF THE DOCTORAL (Ph.D.) DISSERTATION


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# Design of serum albumin-based biocolloidal drug delivery carriers


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# 1. Introduction and aims

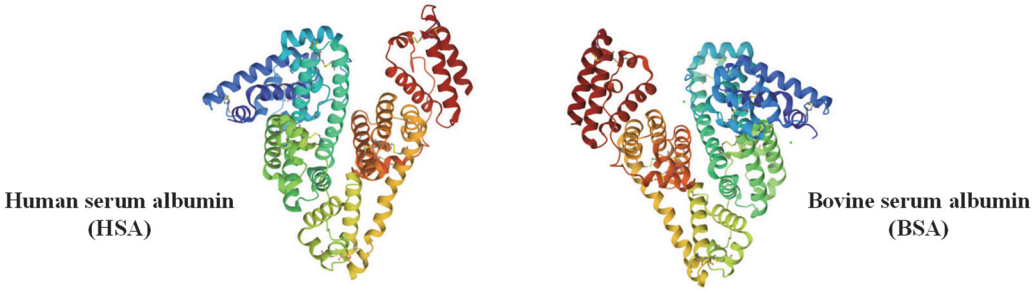
The design of innovative drug delivery carriers is classified among the highly investigated areas of modern colloid chemistry research. The carrier structures potentially used for the targeted delivery and programmed (controlled) release of drugs and the dosage of the active substance required to exert the therapeutic effect contribute to the enhanced bioavailability of compounds achieved. The development of protein-based carriers with biocompatible and biodegradable properties is of particular importance in carrier research. The human serum albumin (HSA) and bovine serum albumin (BSA) are one of the most abundant proteins of the blood plasma. The importance of that heart-shaped serum protein is undeniable, as it is suitable for binding both endogenous and exogenous ligands due to its unique structural features. Considering the advantages of protein-based carriers, the benefits validated the use of albumins in the design of novel (bio)colloidal carrier structures, which contributed to the main motivation of the doctoral research work.

In the course of the doctoral work, the preparation possibilities of simple and complex albumin-based drug delivery systems were mapped, and the effective formulation of various active substances (for instance, non-steroidal anti-inflammatory drugs (NSAIDs), vitamins, and neuroactive drugs) into the carriers featuring controlled drug release was implemented. To highlight the advantages and possibilities of the flow-based technique, the simple and reproducible fabrication of poly(allylamine-hydrochloride) (PAH) polyelectrolyte-stabilized protein-based drug delivery carriers was achieved by a simple and reproducible method with the use of a two-channeled flow chemistry device by tuning the flow parameters. In addition to the investigation of simple protein-based carriers, the preparation of complex BSA/HSA – hyaluronic acid (HyA) drug delivery structures was also investigated by partial charge compensation methods. On the one hand, the nature and extent of interactions between macromolecules and macromolecules/small molecules were examined in detail. By using the protein-polysaccharide complex (bio)colloids, the formulation of an ibuprofen (IBU)-contained drug carrier potentially suitable for the intestine-targeted delivery carrier validated by drug release and special membrane permeability tests is designed. Last but not least, the development of complex protein-based carriers stabilized by vitamin E derivates (TPGS) was investigated. The carriers suitable for the delivery of hydrophobic compounds were fabricated by changing the composition of the macromolecules and the amount of stabilizing agent, resulting in a change in the physical and chemical properties of the carriers.

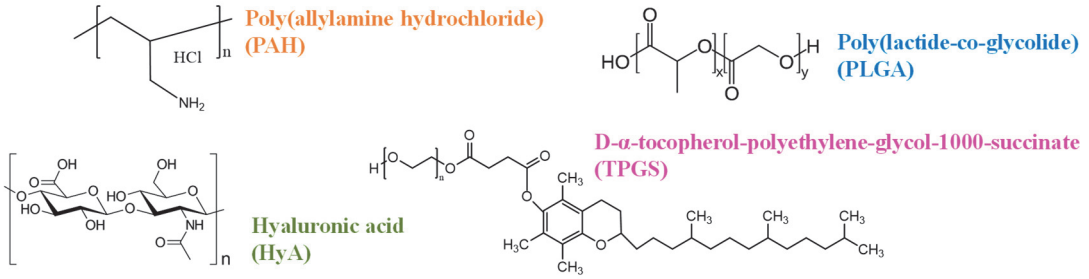
## 2. Methods

For the preparation of each of the different serum albumin-based (bio)colloidal structures, as well as for the stock solutions and buffer, we use Milli-Q distilled water (Millipore, MilliQ Integral3, conductivity 0,055 μS/cm at 25 °C). The applied materials were used without further purification. The macromolecules, stabilizing agents, and drug compounds are presented in **Figure 1**.

### I. Serum albumins (HSA/BSA)




### II. Stabilizing agents (PAH, TPGS), Polysaccharides (HyA), Polymers (PLGA)




### III. Compounds used as model drugs

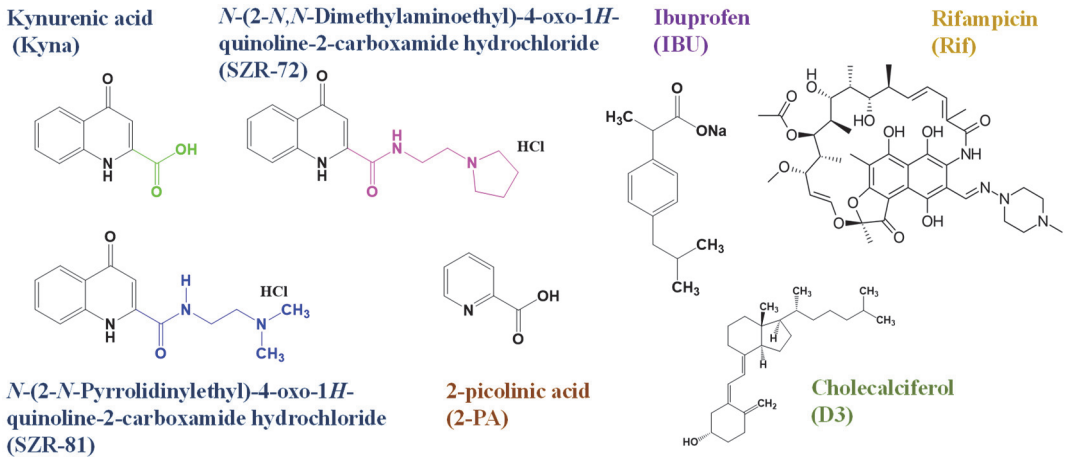



**Figure 1.** The applied macromolecules and model drug compounds *(the monomer units are presented in the figure in the case of PLGA, PAH, and HyA; the structure of BSA and HSA is sourced from the Protein Data Bank (PDB identifier:3V03, 1E78)*).

The following measurement techniques were used to study the size, size distribution, and structural characterization of serum albumin-based drug delivery carriers.[1]

**The measurement techniques used to study interactions between macromolecules/small molecules, and complex macromolecules:**

- Fourier-transformed infrared spectroscopy (*FT-IR; Jasco FT/IR-4700*)
- Surface plasmon resonance spectroscopy (*SPR*, *University of Prague*)
- Circular dichroism spectroscopy (*CD*, *Jasco J1100*)
- Differential scanning calorimetry (*DSC, Mettler-Toledo 822$^e$*)
- Thermogravimetric analysis (*TG, Mettler-Toledo TG/SDTA 851$^e$*)
- Contact angle measurement (*KRÜSS FM40Mk2 Easy Drop*)
- Particle charge detector (*PCD*, *Mütek PCD-04*)
- Rheology (*Anton Paar MCR 301*)
- Isothermal titration calorimetry (ITC, *MicroCal VP-ITC*)

**The measurement techniques utilized for the preparation and characterization of the drug-free and drug-loaded colloidal drug delivery carriers containing serum albumin and, furthermore, for the dissolution study of the encapsulated compounds:**

- Flow techniques (*Syrris Asia Flow* (*Syrris Ltd.))*
- Dynamic light scattering and ζ-potential measurement (*DLS, Horiba SZ-100*)
- Transmission electron microscopy (*TEM, Jeol JEM-1400plus*)
- Turbidimetry (*LP2000 Hanna Ins., Ocean Optics USB4000*)
- Freeze-drying (*Christ Alpha 1-2 LD*)
- UV-Vis spectrophotometry (*Shimadzu UV-1800*)
- High-performance liquid chromatography (*HPLC*, *Agilent 1260 HPLC*)
- Parallel artificial membrane permeability assay (*PAMPA; Rapid Equilibrium Dialysis (RED) device (Thermo ScientificTM, Waltham*)

[1] *The abbreviated name of the given measurement technique and the type of instrument used are also presented in parentheses.*

## 3. Results

**T1. The preparation of protein-based colloidal particles without active compounds and with neuroactive drug molecules can be produced reproductively by flow chemistry techniques [1].**

**T1.1.** Using the continuous flow chemistry technique, the preparation of BSA/PAH delivery carriers possessing a presumed core-shell structure, which function as drug delivery carriers, was fulfilled for the first time. It is verified that by the systematic increase of the flow rates at a constant relative flow rate ($v_1$:$v_2$ = 1:4), the average diameter of the formed carriers can be tuned in the size range of 90–150 nm.

**T1.2.** The neuroactive kynurenic acid and its structural analogue (SZR-72) were formulated into the BSA/PAH carriers, resulting in particles with an average diameter of ~ 150 nm. The drug content (~ 5%) and the encapsulation efficiency (~ 12%) resulted in nearly the same value for both the flow-chemistry technique and the classical wet chemistry technique as well; however, an accelerated release process and a greater amount of the encapsulated drug were released in the case of the particles prepared with the use of flow techniques. Based on the results, the production of HSA/PAH carrier particles is feasible; however, the encapsulation of model drug compounds is less favourable by using lower flow rates, in agreement with the binding of small molecules to the BSA and HSA serum proteins verified by molecular dynamics calculations.

**T2. The (bio)colloids formed by serum protein and hyaluronic acid function as colloidal carrier structures and can be prepared by tuning the pH-dependent interactions between the macromolecules [2-3].**

**T2.1.** The interaction between the serum protein BSA and the high molecular weight polysaccharide at different pH values (pH = 3.5–5.5) was characterized by surface plasmon resonance (SPR), circular dichroism (CD) spectroscopy, rheological, and particle charge detector (PCD) measurements for the first time. Based on the results, we can conclude that the charge of the macromolecules dominantly affects the formation of BSA/HyA (bio)carriers formed by the self-assembly method. The formation of the particles is not favourable at pH above the isoelectric point of BSA (pH ~ 5.1) due to the electrostatic repulsive interaction between the HyA polysaccharide possessing one negative charge per monomer unit ($pK_{HyA}$ ~ 3–4) and BSA with a cumulative negative charge.

**T2.2.** In light of the investigated pH-dependent interactions between the macromolecules, a simple process for the preparation of BSA/HyA and HSA/HyA colloidal carriers was elaborated.

Experimentally verified, the size of the serum-protein-hyaluronic acid colloids based on a simple charge compensation method formed by the self-assembly of macromolecules can be controlled by changing the pH of the medium and the mass ratio between the macromolecules. The size of the carriers, applying the $m_{protein}$:$m_{HyA}$ = 2:1 mass ratio, is ~ 200 nm for both of the serum albumins.

**T3. The formulation of ibuprofen (IBU) can be implemented using both BSA/HyA and HSA/HyA (bio)colloidal carrier particles, where the release of the active ingredient modelling small intestine adsorption is affected by the type of albumin [3].**

**T3.1.** The encapsulation of various active substances (IBU, 2-picolinic acid, KYNA, SZR-72, and SZR-81) possessing diverse effects is examined in the BSA/HyA and HSA/HyA carriers. In the case of IBU, 20 % of drug loading is achieved by applying $m_{IBU}/m_{protein}/m_{HyA}$ = 2:1:0,5, which results in the d ~ 260 nm size of the carriers. Molecular simulation calculations (based on MM/GBSA calculations) confirmed the differences in the protein-drug binding of the different serum albumins.

**T3.2.** By studying the dissolution of the IBU-containing BSA/HyA and HSA/HyA formulations in a medium simulating adsorption in the small intestine (pH = 6.5 (PBS, NaCl, 0.9 %)), we confirmed that the active ingredient can be transferred through the membrane in an accelerated way (3–4 times faster) with the use of albumin-based carriers; therefore, the solubility of the IBU in the aqueous medium can be increased. The BSA-based formulation ensures greater IBU retention, while faster drug release and membrane transport can be achieved by using HSA-based particles.

**T3.3** Applying the same experimental circumstances (pH = 6.5), a significant difference appeared between the apparent surface charge of BSA ($c_{protein}$ = 0,36 mg $mL^{-1}$) and HSA (q = 0,226 ± 0,012 mmol $mg^{-1}$) as revealed by particle charge detection measurements, explaining the results of Thesis point *T3.2.* Specifically, in the case of pH = 6.5, a greater electrostatic repulsion between the negatively charged IBU and the HSA with a greater negative charge can also generate a faster release of the active ingredient. The experimental result can also be supported by molecular simulation calculations, as higher MM/GBSA[2] interaction values can be determined in the BSA-IBU (~ −50–60 kcal/mol) than in the HSA-IBU (~ −40–50 kcal/mol), represented in **Figure 2**.

[2] MM/GBSA: *Molecular mechanics with generalised Born and surface area solvation*

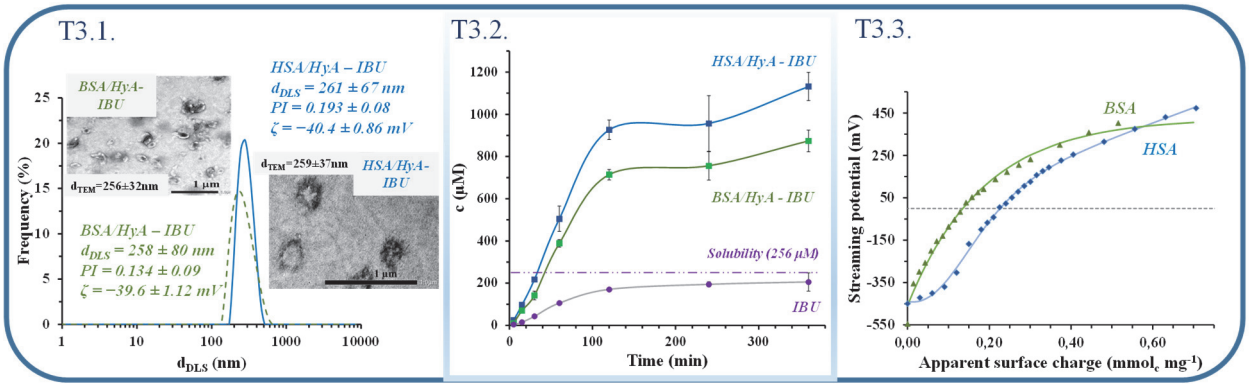



**Figure 2.** The representation of the highlighted results of **T3:** (**T3.1.**) the average hydrodynamic diameter and the morphological characteristics of the prepared IBU-contained BSA/HyA-IBU and HSA/HyA-IBU systems; (**T3.2.**) the dissolution profiles of the unformulated active ingredient and the IBU-containing formulation modelling small intestinal and (**T3.3.**) the net charge difference of the protein (BSA, HSA) determined by particle charge calculations [2,3].

**T4. By combining serum albumin (BSA) and PLGA with different monomer ratios (50:50, 65:35, 75:25) to develop a simple, reproducible preparation process for a non-ionic surfactant (a biocompatible vitamin E derivate), stabilized serum protein/PLGA colloidal particles can be formed. By combining the macromolecules, the hydrophobic character of PLGA can be reduced, which can be further controlled by changing the proportion of monomers [4].**

**T4.1.** The interactions between the BSA-TPGS and the BSA-PLGA are determined using calorimetric (ITC), CD, Fourier-transformed infrared (FT-IR) spectroscopy, and thermoanalytical methods. To optimize the preparation process, the conditions affecting the formability of colloidal nanocarriers (for instance, the mass ratio of the macromolecules and the content of TPGS) were determined experimentally.

**T4.2.** For the first time, we proved that the ”interaction” of the serum albumin protein with the PLGA copolymers of different monomer ratios (lactide:glycolide ratio = 50:50; 65:35; 75:25) can form colloidal particles with an average diameter of d ~ 200 nm in order to ensure their stability. The applicability of a vitamin E derivate, TPGS, with biocompatible properties was also demonstrated. The hydrophobic character of PLGA (contact angle (PLGA50:50) = 56.43°; contact angle (PLGA75:25) = 70.48° (against water)) can significantly be reduced by the formation of complex colloidal particles associated with serum albumins (contact angle (BSA/PLGA50:50) = 36.06°; contact angle (BSA/PLGA75:25) = 38.73° (against water)), as shown in **Figure 3**.

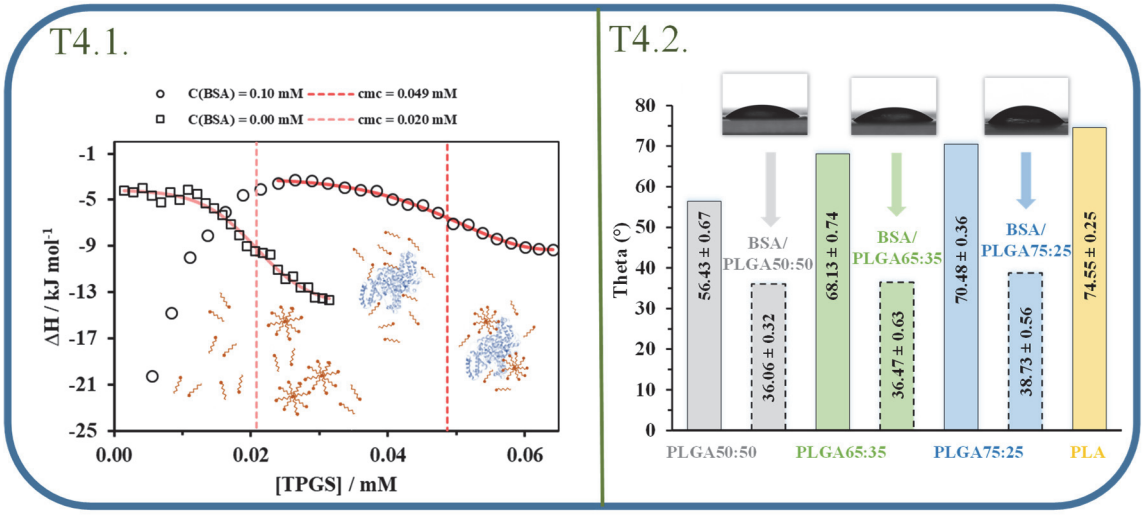



**Figure 3.** The representation of the **T4.1.** and **T4.2.** thesis points: (**T4.1.**) The determination of the cmc value of TPGS by isothermal titration calorimetry and (**T4.2.**) the presentation of the PLGA with different monomer ratios and the BSA/PLGA carrier structures [4].

**T4.3**. The encapsulation of a hydrophobic model drug to the TPGS-stabilized BSA/PLGA particles (d ~150 nm). Our results confirmed that TPGS fulfils a trifold role in the formulation. In addition to its function as a stabilizer of colloidal particles, it is itself a functional vitamin active ingredient, and due to its characteristic structural property (amphiphilic compound), it can also play a dominant role in the solubilization of the hydrophobic model active ingredient.

## 3. Expected consumption of the results

The development of new types of drug delivery carriers is of particular interest in modern pharmaceutical research; hence, there is an ongoing demand for the production of innovative pharmaceutical compounds. The development of novel colloidal drug delivery carriers with biocompatible and biodegradable features is the focus of attention in order to achieve targeted drug delivery carriers with enhanced bioavailability. During my doctoral work, the preparation of various macromolecular drug delivery carriers was fulfilled, demonstrating that the formulation of different active substances can be achieved by a systematic change in the composition and materials of the carriers. The results can contribute to the design of novel drug delivery structures.

# List of publications

## Publications covered in the doctoral work

*Hungarian Scientific Bibliography (MTMT) identifier: 10073570*

[1.] **A. N. Kovács**, N. Varga, Gy. Gombár, V. Hornok, E. Csapó: Novel feasibilities for preparation of serum albumin-based core-shell nanoparticles in flow conditions, Journal of Flow Chemistry, 10 (2020), 497
DOI: 10.1007/s41981-020-00088-4

IF = 3.622; Q1 (2020)

[2.] **A. N. Kovács**, N. Varga, Á. Juhász, E. Csapó: Serum protein-hyaluronic acid complex nanocarriers: Structural characterisation and encapsulation possibilities; Carbohydrate Polymers, 251 (2021) 117047
DOI: 10.1016/j.carbpol.2020.117047

IF = 9.381; D1 (2021)

[3.] **A. N. Kovács**, G. Katona, Á. Juhász, Gy. T. Balogh, E. Csapó: Albumin-hyaluronic acid colloidal nanocarriers: Effect of human and bovine serum albumin for intestinal ibuprofen release enhancement; Journal of Molecular Liquids, 351 (2022) 118614
DOI: 10.1016/j.molliq.2022.118614

IF = 6.633; Q1 (2022)

[4.] **A. N. Kovács**, N. Varga, J. Bogner, Á. Juhász, E. Csapó: Enhancing the hydrophilicity of poly(lactic-co-glycolic acid) with serum albumin by creating colloidal drug carriers; Journal of Molecular Liquids, 398 (2024) 124271
DOI: 10.1016/j.molliq.2024.124271

IF = 6.165; Q1 (2024)

**∑ IF = 25.801**

## Additional publications

[5.] V. Hornok, Á. Juhász, G. Paragi, **A. N. Kovács**, E. Csapó: Thermodynamic and kinetic insights into the interaction of kynurenic acid with human serum albumin: Spectroscopic and calorimetric approaches, Journal of Molecular Liquids, 313 (2020), 112869
DOI: 10.1016/j.molliq.2020.112869 (2020)

IF = 5.075; Q1 (2020)

[6.] N. Varga, L. Seres, **A. N. Kovács**, Á. Turcsányi, Á. Juhász, E. Csapó: Serum albumin/hyaluronic acid nanoconjugate: Evaluation of concentration-dependent structural changes to form an efficient drug carrier particle, International Journal of Biological Macromolecules, 220 (2022) 1523
DOI: 10.1016/j.ijbiomac.2022.09.125

IF = 8.025; Q1 (2022)

[7.] V. Hornok, K. W. K. Amin, **A. N. Kovács**, Á. Juhász, G. Katona, Gy. T. Balogh: E. Csapó: Increased blood-brain barrier permeability of neuroprotective drug by colloidal serum albumin carriers, Colloids and Surfaces B: Biointerfaces, 220 (2022) 112935
DOI: 10.1016/j.colsurfb.2022.112935

IF= 5.999; Q1 (2022)

**Number of independent citations (covered in the doctoral theses): 32**

**Number of total (independent) citations: 51**

$$\frac{\sum \textbf{IF} = \textbf{25.801}}{\sum\sum \textbf{IF} = \textbf{44.900}}$$

## Lectures and posters related to the doctoral work

1. **A. N. Kovács**: Serum albumin/hyaluronic acid complex nanocarriers; structural characterisation and encapsulation possibilities, 13th International Conference on Nanomaterial, 2021, Brno, Czech Republic (lecture in English)

2. **A. N. Kovács**: Serum albumin/hyaluronic acid complex nanocarriers; structural characterisation and encapsulation possibilities, 18th European Student Conference, 2022, Szeged, Hungary (lecture in English)

3. **A. N. Kovács**, N. Varga, G. Katona, Á. Juhász, Gy. T. Balogh, E. Csapó: Protein-polysaccharide complex colloidal carriers: synthesis, optimization and applicability possibilities, 11th International Colloids Conference, Lisbon, Portugal, 2022 (poster)

4. **A. N. Kovács**, N. Varga, Á. Juhász, E. Csapó: Protein-based complex colloidal structures: design of effective drug carrier particles, 12th International Colloids Conference, Mallorca, Spain, 2023 (poster)

5. **Kovács N. A.**, Hornok V., Csapó E.: Szérum albumin alapú kompozitok előállítási lehetőségeinek vizsgálata, Szeged, XLII. Kémiai Előadói Napok, 2019, Szeged, Magyarország (lecture in Hungarian)

6. **Kovács N. A.**, Varga N., Juhász Á., Csapó E.: Szérum albumin-hialuronsav alapú komplex hatóanyaghordozó rendszerek. XLIII. Kémiai Előadói Napok 2020, Szeged, Magyarország (lecture in Hungarian)

7. **Kovács N. A.**, Csapó E.: Fehérje-alapú kolloidális gyógyszerhordozók modellezése „kvázi” 2D és 3D technikákkal, XXIV. Tavaszi Szél Konferencia 2021, Miskolc, Magyarország (lecture in Hungarian)

## Additional presentations

8. P. Bélteky, V. E. Resch, **A. N. Kovács**, I. Y. Tóth, A. Rónavári, Z. Kónya: Colloidal stability of silver nanoparticles in biorelevant conditions, Szeged, 8th Szeged International Workshop on Advances in Nanosciences, 2018, Szeged, Hungary (poster)

9. Á. Juhász, **A. N. Kovács**, E. Csapó: Thermodynamic evidence for a predicted intermolecular salt-bridge between kynurenic acid and biomimetic sensor surface, 9th International Colloids Conference, 2019, Barcelona-Sitges, Barcelona (poster)

10. V. Varga, H. Szokolai, **A. N. Kovács**, N. Varga, E. Csapó: Investigation of different human serum albumin-based composites for kynurenic acid drug delivery, 9th International Colloids Conference, 2019, Barcelona-Sitges, Spain (poster)