AGGREGATION BEHAVIOR OF SILVER NANOPARTICLES IN VARYING SIZES AND SURFACE CAPPING UNDER BIORELEVANT CONDITIONS AND ITS EFFECT ON NANOPARTICLE TOXICITY

PH. D. THESIS

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INTRODUCTION AND OBJECTIVES

Due to their excellent and precisely tunable optical characteristics, furthermore, biological and catalytic activities silver nanoparticles are one of the most important nanosized materials. Through their cytotoxic and antimicrobial properties, nanosilver can be found in numerous commercial products, and are used in various biomedical approaches as antimicrobial agents, drug delivery systems, biosensors, chemotherapeutic agents, or amplifiers for molecular imaging techniques. The unique properties demonstrated by nanomaterials – and therefore, by silver nanoparticles as well – are attributed to their large specific surface area, which inevitably prompts a large amount of surface energy. To reach thermodynamic energy minimum, these systems aim to decrease their surface energy, which is generally achieved by attractive forces among the particles, and the resulting aggregation may, in turn, reduce, or even completely suppress the desired characteristics originally provided by particle size. Unfortunately, most scientific contributions addressing the biological application of silver nanoparticles do not consider aggregation, even though the structure and composition of living organisms and environmental systems are far from ideal regarding the colloidal stability of nanoparticles. Moreover, aggregation is affected by various factors relevant to bio-nano research, for instance, the size and surface capping of nanoparticles.

Throughout our research, the effects of nanoparticle size and capping mechanism were investigated on the colloidal stability of silver nanoparticles in biorelevant conditions. Changes caused by size were assessed through electrostatically stabilized citrate-capped silver nanoparticles of 10, 20, and 50 nm average diameters, while the effects of stabilizing agents were investigated by comparing the beforementioned 10 nm citrate capped nanosilver with polyvinyl pyrrolidone and green tea extract stabilized silver colloids of the same size, corresponding to steric and electrosteric stabilization, respectively. After the aggregation behavior experiments consisting of dynamic light scattering and zeta potential measurements, furthermore, ultraviolet-visible light spectroscopy, the direct influence of aggregation on nanoparticle toxicity was also studied with the help of *in vitro* viability assays performed on human cell lines and microbes.

EXPERIMENTAL SECTION

Citrate-capped silver nanoparticles of 10 nm average diameter (AgNP@C_{10}) were synthesized through the chemical reduction of silver nitrate with sodium borohydride in the presence of trisodium citrate. During the reaction, 75 mL 9 mM aqueous solution of trisodium citrate was heated to 70 °C, in which 2 mL 1 w/v% $AgNO_3$ solution, and finally 2 mL of 0.1 w/v% freshly prepared $NaBH_4$ solution was added. The resulting golden-brown colloid was stirred at 70 °C for 1 hour, then it was left to cool to room temperature.

Silver nanoparticles of 20 and 50 nm diameters were prepared through consecutive seed-mediated growth steps. Synthesizing the sol containing 20 nm particles (AgNP@C_{20}), 10 mL of AgNP@C_{10} was added to 90 mL of 7.6 mM aqueous citrate solution at 80 °C and was stirred for one hour at this temperature. Finally, the sample containing the largest particles (AgNP@C_{50}) was prepared with the same method, but this time 10 mL of AgNP@C_{20} was used as seed solution.

Polyvinyl pyrrolidone stabilized particles were formulated in a 95 mL polymer solution containing 0.17 g PVP on 70 °C, in which 2 mL 1 w/v% $AgNO_3$, and 2 mL of 0.1 w/v% freshly prepared $NaBH_4$ solutions were added, followed by 1 hour of vigorous stirring at the same temperature.

For the synthesis of green tea stabilized 10 nm nanoparticles (AgNP@GT_{10}), green tea extract was brewed by adding 2 g of shredded green tea leaves in 100 mL deionized water at 80 °C, which was filtered by a 0.45 μm pore size nylon membrane after 20 minutes of stirring. The particles were formed through the mixing of the tea extract with 0.1 M $AgNO_3$ solution in a 2:1 volume ratio at room temperature. After 24 h of stirring, the product was once again filtered by a 0.22 μm syringe filter.

The success of each synthesis was verified with transmission electron microscopy. The size, dispersity, and morphology of the particles were assessed through the evaluation of 15 representative images, while their crystalline characteristics were investigated by electron diffraction. The characteristic UV-Vis spectra of the samples, generated by the surface plasmon resonance of the nanoparticles were used to gather further proof regarding their chemical composition.

The aggregation behavior of silver nanoparticles was examined by measuring three distinct parameters: dynamic light scattering (DLS) was used to follow the changes of the average hydrodynamic diameter within the samples, zeta potential (ζ-potential) measurements described the colloidal stability of the particles, finally changes observed in the characteristic

light absorbance spectra of the colloids indicated surface interactions, furthermore alterations in aggregation grade, and in certain cases chemical stability.

The biorelevant conditions were set in samples of 5 mL in volume with 10 ppm nanoparticle concentration since in this concentration the UV-Vis absorbance maximum of each sample was around 1. Throughout the experiments the effect of pH (3, 5, 7.2, and 9), the concentration of sodium chloride (10, 50, and 150 mM), glucose (3.9 and 6.7 mM), and glutamine (4 mM) on the colloidal stability of silver nanoparticles were investigated. In addition, modeled via the *in vitro* cell culture components DMEM (*Dulbecco's Modified Eagle's Medium*) and FBS (*fetal bovine serum*), we also examined how nanoparticle aggregation would occur in more complex biological conditions. Each experiment consisted of a 24-hour procedure, with measurements taken at the 0, 1.5, 3, 6, 12, and 24-hour marks, upon which the average hydrodynamic diameter, zeta potential, and characteristic light absorbance spectrum of the samples were measured.

The subsequent in vitro studies consisted of MTT assays performed on human cancer (A549, DU145, HeLa) and non-cancerous (MRC-5, HaCaT) cell lines, as well as of microdilution experiments on fungal (*C. neoformans*), Gram-positive (*B. megaterium*) and Gram-negative (*E. coli*) microbial strains. Firstly, the IC_{50} and MIC values of the silver colloids were defined for the human cell lines and microbes, respectively. The relationship between aggregation grade and toxicity was ultimately measured by treating cells with silver colloids in the respective IC_{50} or MIC concentrations mixed with 150 mM NaCl for certain time intervals to induce the various aggregation grades measured throughout the colloidal stability experiments.

NOVEL SCIENTIFIC RESULTS

T1: Using an innovative approach, a novel experimental protocol was created by combining chemical and biological methodologies, allowing us to investigate both nanoparticle aggregation and its biological consequences.

1.1 We reported for the first time a comprehensive protocol consisting of average hydrodynamic diameter and zeta potential, furthermore UV-Vis spectroscopy measurements, through which the aggregation behavior of silver nanoparticles under biorelevant conditions can be assessed. We demonstrated that the combined discussion of these results can inform us about certain features of silver nanoparticle colloids such as surface interactions, colloidal and chemical stability.

1.2. We performed unique cell viability and microdilution experiments for the first time in the relevant scientific literature, where the investigated variable was nanoparticle aggregation grade instead of concentration, illustrating the profound connection between silver nanoparticle toxicity and colloidal stability.

T2: We demonstrated that smaller nanoparticle diameters lead to decreased long-term toxicity through weaker biorelevant colloidal stability, highlighting the complex biological significance of nanoparticle size.

2.1 Through the experimental results we have verified, that the increased size of electrostatically stabilized silver nanoparticles achieved via seed-mediated growth not only increased their colloidal stability but their resistance against several aggregation-inducing conditions was also elevated. The greater biorelevant stability of larger particles was displayed at certain pH and NaCl concentrations. Furthermore, in the presence of DMEM - corresponding to roughly 65 mM ionic strength - silver nanoparticles of the three AgNP@C colloids demonstrated distinct and inversely proportional aggregation grade with primer particle size.

2.2 The increased colloidal stability of larger nanoparticles proved to be significant regarding their prolonged toxicity as well. While the colloid containing the smallest particles ($AgNP@C_{10}$) lost its biological activity entirely on every cell line and microbial strain, apart from A549, by 24 hours, the largest, 50 nm particles of $AgNP@C_{50}$ retained a certain degree of toxicity in every experimental setup.

2.3 Even though the IC_{50} and MIC experiments we performed supported the claim of the general literature proposing that the native biological activity of silver nanoparticles increases with decreasing particle size, our aggregation related *in vitro* toxicity experiments demonstrated that in biologically relevant environments the stronger toxic effect of smaller nanoparticles might not be a feasible advantage due to large-scale aggregation. Based on these observations, instead of aiming for the smallest nanoparticles possible, establishing an optimal nanoparticle size would be more advantageous during the biomedical application of silver nanoparticles.

T3: We highlighted how the stabilizing mechanisms on silver nanoparticles affect their colloidal stability and induce biologically relevant discrepancies.

3.1 While citrate-capped, electrostatically stabilized silver nanoparticles are the most readily available and most utilized nanosilver systems, according to our results this stabilization approach resulted the most vulnerable samples when placed in biorelevant conditions, demonstrating weak colloidal stability both in acidic pH and physiological NaCl concentration, leading ultimately to the formation of micron-sized aggregates.

3.2 In the case of sterically stabilized silver nanoparticles with polyvinyl pyrrolidone capping, remarkable colloidal stability was observed. Apart from a mildly acidic milieu (pH 5) – where moderately increased hydrodynamic diameters were observed due to the slightly elevated H^+ content – aggregate growth within this system was virtually negligible under any biorelevant condition. Our experiments also demonstrated that PVP coverage was unable to chemically stabilize the surface of nanoparticles, leading to silver chloride precipitation in the presence of elevated Cl^- content, expressed through the decrease of ζ-potential and the increase of the UV-Vis spectral baseline of the nanoparticles.

3.3 Silver nanoparticles capped by green tea extract were stabilized through electrosteric interactions, demonstrating a behavior that showed commonalities with both previous nanosilver sols. The changes detected in the colloidal stability of AgNP@GT_{10} induced by biorelevant conditions showed similar trends that were observed in the citrate-capped electrostatic system, however, owing to the large biomolecules present in green tea that provided strong steric interactions among the nanoparticles, similarly to PVP, only mild aggregation occurred even under the harshest conditions. In addition to the strong colloidal stability of AgNP@GT_{10}, the nanoparticles proved to be chemically stable as

well, indicating that within our experimental conditions, electrosteric stabilization provided both colloidal and chemical stability simultaneously.

3.4 We proved that the altered aggregation behavior of silver nanoparticles of different stabilizing mechanisms affects their toxicity as well. Colloidal stability proved to be essential for the longevity of the toxic effects attributed to silver nanoparticles. While particles prone to aggregation, like AgNP@C_{10}, virtually lost their biological activity in most of our *in vitro* experiments, the samples AgNP@PVP_{10} and AgNP@GT_{10} were able to retain their toxicity. While the chemical degradation of the silver nanoparticles stabilized by PVP did not cause changes in their activity, the detected precipitation can pose severe consequences in real biomedical applications. In conclusion of our experimental data, silver nanoparticles stabilized by green tea extract demonstrated the highest safety and effectiveness in biologically relevant environments.

T4: We have described the positive effect of biomolecular coronas on the chemical and colloidal stability of nanoparticles.

4.1 We proved, that biomolecular coronas formed in systems containing 5 v/v% fetal bovine serum possess the ability to improve the colloidal stability of silver nanoparticles prone to aggregation, regardless of particle size. This property of biomolecular coronas was the most prominent in AgNP@C_{50}, where the combined effect of the larger particle size and corona formation could prevent the aggregation of nanoparticles even at elevated electrolyte concentrations.

4.2 Biomolecular coronas are capable to provide chemical protection, as was observed for the AgNP@PVP_{10} colloid. In the case of chemically degrading nanoparticles, the addition of FBS caused unique UV-Vis absorption peaks attributed to the appearance of particle clusters. This indicated that upon the initial steps of silver nanoparticle degradation fragmentation occurs at a certain degree, however, the biomolecules present in the environment construct protective shells around the newly formed clusters, resulting in delayed bulk precipitation of nanosilver.

PUBLICATIONS RELATED TO THE THESIS

1. **Silver nanoparticles: aggregation behavior in biorelevant conditions and its impact on biological activity**

   Bélteky, P., Rónavári, A., Igaz, N., Szerencsés, B., Tóth, I. Y., Pfeiffer, I., Kiricsi, M., Kónya, Z.

   Int J Nanomed, 2019, 14: 667-687

   DOI: 10.2147/IJN.S185965

   $IF_{2019/2020}$ = 5.115 Independent citations: 25

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   Int J Nanomed, 2021, 16: 3021-3040

   DOI: 10.2147/IJN.S304138

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3. **Polyvinylpyrrolidone coated silver nanoparticles - The colloidal, chemical, and biological consequences of steric stabilization under biorelevant conditions**

   Rónavári, A., **Bélteky, P.**, Boka, E., Zakupszky, D., Igaz, N., Szerencsés, B., Pfeiffer, I., Vágvölgyi, Cs., Kónya, Z., Kiricsi, M.

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CONFERENCE CONTRIBUTIONS RELATED TO THE THESIS

1. **The effect of biological conditions on silver nanoparticle toxicity: just how important nanoparticle aggregation really is?**

   Bélteky, P., Rónavári, A., Boka, E., Zakupszky, D., Igaz, N., Szerencsés, B., Tóth, I. Y., Pfeiffer, I., Kiricsi, M., Kónya, Z.

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2. **A felületi stabilizáció mechanizmusának szerepe az ezüst nanorészecskék aggregációs viselkedésére bioreleváns körülmények között**

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3. **Aggregation behavior of silver nanoparticles in biorelevant conditions**
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   FEMS Junior Euromat Conference, Budapest, 2018 (előadás)
4. **Colloidal stability of silver nanoparticles in biorelevant conditions**
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   SIWAN8: 8th Szeged International Workshop on Advances in Nanoscience, Szeged, 2018
5. **Silver nanoparticles in lifelike environments**
   Bélteky, P., Kovács, D., Igaz, N., Kiricsi, M., Kukovecz, Á., Kónya, Z.
   SIWAN 7: 7th Szeged International Workshop on Advances in Nanoscience, Szeged, 2016

UNRELATED PUBLICATIONS

1. **Mechanochemical synthesis of the NiSn, CuSn bimetallic and NiCuSn trimetallic nanocomposites using various types of additives**
   Musza, K., Szabados, M., Ádám, A.A., **Bélteky, P.**, Kónya, Z., Kukovecz, Á., Sipos, P., Pálinkó, I.
   J Solid State Chem, 2021, 293: 121756
   DOI: 10.1016/j.jssc.2020.121756
   $IF_{2019/2020}$ = 2.726 Independent citations: -
2. **Size-dependent activity of silver nanoparticles on the morphological switch and biofilm formation of opportunistic pathogenic yeasts**
   Szerencsés, B., Igaz, N., Tóbiás, Á., Prucsi, Zs., Rónavári, A., **Bélteky, P.**, Madarász, D., Papp, Cs., Makra, I., Vágvölgyi, Cs., Kónya, Z., Pfeiffer, I., Kiricsi, M.
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   $IF_{2019/2020}$ = 2.989 Independent citations: -
3. **Controlled Size Reduction of Liquid Exfoliated Graphene Micro-Sheets via Tip Sonication**
   Di Berardino, C., **Bélteky, P.**, Schmitz, F., Lamberti, F., Menna, E., Kukovecz, Á., Gatti, T.,
   Crystals, 2020, 10(11): 1049
   DOI: 10.3390/cryst10111049
   $IF_{2019/2020}$ = 2.404 Independent citations: -

4. **Synergistic Radiosensitization by Gold Nanoparticles and the Histone Deacetylase Inhibitor SAHA in 2D and 3D Cancer Cell Cultures**

   Igaz, N., Szőke, K., Kovács, D., Buhala, A., Varga, Z., **Bélteky, P.**, Rázga, Zs., Tiszlavicz, L., Vizler, Cs., Hideghéty, K., Kónya, Z., Kiricsi, M.

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   DOI: 10.3390/nano10010158

   $IF_{2019/2020}$ = 4.324 Independent citations: 1

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   Kovács, D., Igaz, N., Marton, A., Rónavári, A., **Bélteky, P.**, Bodai, L., Spengler, G., Tiszlavicz, L., Rázga, Zs., Hegyi, P., Vizler, Cs., Boros, I. M., Kónya, Z., Kiricsi, M.

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   DOI: 10.1186/s12951-020-0576-x

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   $IF_{2019/2020}$ = 4.872 Independent citations: -

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   DOI: 10.1016/j.chemosphere.2020.126419

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   Sipos, B., Szabó-Révész, P., Csóka, I., Pallagi, E.; Dobó, D. G., **Bélteky, P.**, Kónya, Z., Deák, Á., Janovák, L., Katona, G.

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   $IF_{2019/2020}$ = 4.421 Independent citations: -

9. **Mechanochemical and wet chemical syntheses of CaIn-layered double hydroxide and its performance in a transesterification reaction compared to those of other Ca2M(III) hydrocalumites (M: Al, Sc, V, Cr, Fe, Ga) and Mg(II)-, Ni(II)-, Co(II)- or Zn(II)-based hydrotalcites**

   Szabados, M., Ádám, A. A., Traj, P., Muráth, Sz., Baán, K., **Bélteky, P.**, Kónya, Z., Kukovecz, Á., Sipos, P., Pálinkó, I.

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   $IF_{2019/2020}$ = 7.888 Independent citations: 2

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    Vágvölgyi, M., **Bélteky, P.**, Bogdán, D., Nové, M., Spengler, G., Latif, A. D., Zupkó, I., Gáti, T., Tóth, G., Kónya, Z., Hunyadi, A.

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    DOI: 10.3389/fphar.2020.552088

    $IF_{2019/2020}$ = 4.225 Independent citations: -

11. **Effects of medium and nickel salt source in the synthesis and catalytic performance of nano-sized nickel in the Suzuki-Miyaura cross-coupling reaction**

    Ádám, A. A., Szabados, M., Musza, K., **Bélteky, P.**, Kónya, Z., Kukovecz, Á., Sipos, P., Pálinkó, I.

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12. **Nanotechnológia a környezettudományban - nanorészecskék kölcsönhatása a környezettel**

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    Kozma, G., Puskás, R., Papp, I. Z., Bélteky, P., Kónya, Z., Kukovecz, Á.

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    DOI: 10.1016/j.carbon.2016.05.005

    IF_{2016} = 6.337 Independent citations: 3

SCIENTOMETRIC DATA

Sum of peer-reviewed publications:	17	In relation to the thesis:	2
Cumulative impact factor:	75.009	In relation to the thesis:	10.23
Sum of independent citations:	114	In relation to the thesis:	25