

Antitumor Silver-Lipid Nanoparticles for Targeted Tumor Therapy

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1 Introduction and literature background

1.1 Cancer

Cancer is a multifaceted disease, where cells acquire the possibility of proliferating and expanding arbitrarily. This behaviour is believed to be driven by genetic mutations, accompanied with epigenetic modulations. Generally, it is treated by conventional strategies, which include surgery, radiation, and chemotherapy. Unfortunately, chemotherapeutics cause generalized systemic toxicity, radiation affects surrounding healthy tissues, and surgery is invasive and unable to completely remove tumor tissues. In this context, nanotechnology and nanoscience approaches are increasingly contributing to this fight by creating novel strategies for treating tumors, while minimizing harm to healthy tissues.

1.2 Nanoscience

Nanoscience today is concerned with the study of structures and molecules at the nanometre scale, typically between 1 and 100 nm. Nanoparticles (NPs) show exceptional capabilities, making them good candidates to treat cancer. Such include NP ability to passively accumulate in tumor. Also, their surface could be functionalized with targeting ligands. Among NPs, silver nanoparticles (AgNPs) have attracted attention for their biomedical applications.

1.2.1 Silver nanoparticles (AgNPs)

AgNPs are inorganic, metallic NPs (MNPs) composed of elemental silver in nanoscale form. They are especially well-known for their optical and biocidal properties. It is worth mentioning that they enhance the efficacy of conventional chemotherapeutic agents.

It has been proven that AgNPs could employ their cytolytic effect through the intracellular release of Ag^+ via the so-called “*Trojan Horse*” mechanism, where Ag^+ ions generate reactive oxygen species (ROS) leading to oxidative stress (OS). Furthermore, it is worth noting that AgNPs can trigger toxicity via an Ag^+ -independent mechanism.

1.2.1.1 Photothermal properties of AgNPs

AgNPs are also well-known for having surface plasmon resonance (SPR), which allows efficient conversion of light into localized heat upon irradiating AgNPs with a light source. This photothermal effect extends AgNP application against cancer into the photothermal therapy (PTT) by triggering hyperthermia and causing cell death.

1.2.1.2 AgNPs limitations

The mononuclear phagocyte system (MPS) recognizes AgNPs as foreign bodies leading to an immune response, causing “off-target effects” in healthy tissues. This is accompanied by a reduced availability in tumor site. Furthermore, treating mammalian cells with AgNPs trigger OS, DNA damage, and apoptotic pathway activation. Additionally, AgNPs can react with biofluid components such as electrolytes, affecting their own physicochemical properties, thereby compromising their stability. As a result, AgNP toxicity fluctuates and becomes inconsistent. A possible way to overcome over these limitations involve delivering AgNPs using lipid carriers.

1.2.2 Lipid drug delivery systems

Lipid-based drug delivery systems are NP-scale particles, composed of cell-like layer/s and widely employed as carriers to deliver active components. These systems include liposomes and lipid coatings. They typically consist of phospholipids (PLs).

Liposomes are spherical artificial nano systems made of PLs and/or other amphiphilic lipids. They usually include one or more bilayer membranes, forming an aqueous core/cores depending on liposome type. PL bilayers closely resemble natural cell membranes, enabling efficient interaction with cells while minimizing adverse responses and improving compatibility.

1.2.3 Lipid–Metallic Nanoparticle Formulations

The combination of lipid carriers and MNPs represents a promising strategy to integrate the unique properties of both systems. With respect to liposome-encapsulated AgNPs, only a limited number of studies have explored the use of liposomes as a vehicle for AgNPs to evaluate their antineoplastic activity, and these investigations have been restricted to conventional 2D models. In these reports, the liposome formulations were not subjected to purification steps to remove free, unencapsulated AgNPs, making it difficult to distinguish the specific contribution of the encapsulated form that of the free NPs. As a result, the genuine therapeutic potential and mechanistic effects of purified liposomes remain unclear.

Also, coating AgNPs with a hybrid lipid–thiol layer exhibited markedly reduced toxicity in embryonic zebrafish models and also improved AgNP stability and controlled aggregation. Despite these promising results, this coating strategy has not yet been tested in mammalian, tumor cells or *ex vivo* models, which are one of the main focuses of the present study.

2 Aims

2.1 To make and characterize liposome-encapsulated AgNPs (LAGs)

- To assess how lipid composition influences the physicochemical properties.
- To evaluate the suitability of LAGs for biological applications.
- To investigate the passive acute and long-term toxic activity of the most stable LAGs and how encapsulating AgNPs within liposomes affects AgNP toxicity either toward healthy or tumor cells in 2D models.
- To evaluate the antiinvasive potential of LAG formulations in a physiologically relevant 3D context.

2.2 To make and characterize targeted-, lipid-coated AgNPs

- To achieve oxidation-resistant, stable, and biocompatible NPs.
- To assess the shielding capacity of the hybrid lipid coat against aggressive environment.
- To explore the therapeutic effects of coating on AgNPs toxicity toward 2D health control and GBM cell.
- To check if using AgNPs as a photothermal agent (PTA) would improve their effectiveness toward tumor cells.
- To enhance cell-specific active targeting.

3 Material and Methods

3.1 Liposome-encapsulated AgNPs

AgNP synthesis and characterization: Uncapped AgNPs (AgNPs-u) and citrate-capped AgNPs (AgNPs-c) were prepared by chemical reduction, then characterized by ultraviolet–visible (UV–Vis) spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM).

Preparation of liposomes: Liposomes with different lipid compositions (**Table 1**) were prepared using the conventional thin-film hydration method. The dried lipid films were hydrated with AgNPs at room temperature to form MLVs. The resulting suspensions were subjected to extrusion through 100 nm polycarbonate membranes.

Table 1. Different formulas of liposomes.

Lipid type	Molar ratio
1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)	
DOPC/ 1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DOPG)	1/1
DOPC/DOPG/Sphingomyelin (SM)	1/1/1
DOPC/DOPG/Cholesterol (Cho)/(SM)	1/1/1/1
DOPC/DOPG/(Phosphatidylinositol 4,5-biphosphate) PIP₂	40/55/5
DOPC/DOPG/ 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)/PIP ₂	20/20/55/5

Liposome purification and characterization: Purification of liposomes from unencapsulated AgNPs was performed using a size exclusion chromatography (SEC) approach with Sephadex G-200. LAg properties were evaluated by DLS, TEM and X-Ray diffraction. Also, the encapsulation efficiency (EE) was measured by mass spectrometry. Additionally, density gradient centrifugation was used to separate empty liposomes, then fractions were analysed.

LAg leakage assay: Stability of liposomes in bio medium was assessed by encapsulating carboxyfluorescein (CF), then incubating LAgS with DMEM and DMEM + FBS at 37 °C.

LAg 2D toxicity: The cytotoxic effects of AgNPs and LAgS were evaluated in A375, RPMI7951, and HEK293 cells over 48 h using the MTT assay. While the clonogenic potential of A375 and RPMI7951 melanoma cells following the treatment was assessed using a colony formation assay.

3D Spheroid Invasion Assay: The invasive behaviour of melanoma cells in a 3D context was evaluated using a spheroid invasion assay. A375 and RPMI7951 spheroids were embedded into a Matrigel matrix and treated with free AgNPs and LAgS and invasion was then monitored.

3.2 Hybrid lipid-coated triangular AgNPs

Lipid-coated triangular AgNPs: Citrate-stabilized triangular AgNPs were synthesized by the chemical reduction method in the presence of H₂O₂. Then AgNPs were coated with sodium oleate (SOA), dipalmitoylphosphatidylcholine (DPPC) and hexane thiol (HT) yielding the final AgNPs–SOA–DPPC–HT formulation. AgNPs were subsequently characterized by UV–Vis spectroscopy and TEM. This was followed by stability assessment using KCN etching test.

Photothermal potential of lipid coated triangular AgNPs: AgNP suspensions in DMEM were exposed to 808 and 850 nm lasers at a power output of 2W for 6 min. The temperature was continuously monitored during irradiation.

Cytotoxicity of triangular AgNPs: Toxicity of triangular AgNPs against U87, U251 and HEK was evaluated using the MTT assay. Cells were treated with AgNPs followed by laser radiation.

Targeting of triangular AgNPs: Chlorotoxin (CTX) and epidermal growth factor (EGF) were produced through recombinant DNA technology, then conjugated to LUVs by including DSPE-PEG2000-COOH in the bilayer formulation, while the hydrophobic fluorescent dye Dil was incorporated to track AgNP uptake. 3D spheroids were treated with either untargeted or targeted AgNPs and fluorescence imaging was performed.

Ex vivo targeting of human glioma slices: Human glioma tissue sections were incubated with targeted lipid-coated AgNPs, then fluorescence imaging was performed.

4 Results

4.1 Liposome-encapsulated AgNPs

AgNP characterization

AgNPs-u and AgNPs-c showed SPR peaks in the UV-Vis spectra. TEM images directly confirmed the spherical uniform morphology, while DLS measurements further revealed hydrodynamic diameters of 45.0 nm for AgNPs-u and 32.4 nm for AgNPs-c, with Zeta potential values of -27.0 mV and -41.3 mV, respectively.

Encapsulation of AgNPs in liposomes: LAg purification was achieved using SEC, which has not previously done for LAg. Larger LAg were eluted. In contrast, free AgNPs remained trapped near the top of the column. This was confirmed by DLS measurements, by comparing unpurified (had two peaks) and purified LAg samples (had only one peak). TEM images of purified preparations showed only encapsulated NPs, with no evidence of free AgNPs, across all tested purified formulations. Additionally, XRD spectra proved the presence of AgNPs.

LAg properties and biostability: Encapsulation generally resulted in a more negative values of Zeta potential implying an enhanced colloidal stability of LAg. Based on DGC, the density of the vesicles increased upon NP encapsulation and a substantial proportion of LAg sedimented into denser layers of the gradient. Sedimentation behaviour of free AgNPs and liposome-encapsulated AgNPs could be readily distinguished by DGC.

Biostability of LAg: In isotonic medium, leakage was observed for neutral and highly negatively charged LAg. The addition of FBS revealed further differences. Nevertheless, PC/PG-, PC/PG/SM-, and PC/PG/SM/Chol-LAg consistently demonstrated the highest stability under serum conditions.

Purified LAgS suppress tumor cell viability: Both LAgS and free AgNPs reduced the viability of A375 and RPMI7951 cells in a coating agent-dependent method. Encapsulating AgNPs either preserved or improved AgNP efficacy. It is noteworthy that LAg formulations were less toxic than free AgNPs to non-cancerous HEK293 cells. Also, the treatment dramatically inhibited clonogenic potential of both cell lines confirming that LAgS strongly impair the long-term survival and proliferative capacity of melanoma cells.

Purified LAgS inhibit 3D tumor spheroid invasion: Both types of free AgNPs and LAgS reduced invasive growth of A375. While RPMI7951 was not significantly affected by AgNPs-u, but only AgNPs-c and LAg-c.

4.2 Lipid-coated AgNPs

Triangular AgNP characterization: Bare AgNPs revealed a pronounced SPR peak within the NIR region. Following lipid functionalization, coated AgNPs showed preservation of the SPR peak, while TEM images showed the triangular morphology.

Stability of hybrid lipid-coated AgNPs against etching with KCN: AgNPs–SOA–DPPC–HT formulation was the only formula which remained stable under etching conditions.

Photothermal potential of AgNPs: Illuminating AgNP suspensions in cell culture medium resulted in a measurable temperature increase over time in a linear way with AgNP ratio.

Cytotoxic effects of hybrid lipid-coated AgNPs: Coating AgNPs with a lipid layer significantly reduced their toxic effects toward healthy HEK293 cells. At the same time, coated AgNPs retained or improved cytotoxic activity against U87 and U251 cells. In contrast, applying PTT markedly enhanced the toxic effect toward both cell lines.

Targeting of lipid-coated AgNPs to 3D GBM spheroids: Labelled and targeted AgNPs showed a markedly higher fluorescence signal with both types of tumor spheroids as compared to untargeted controls. Quantitative analysis of fluorescence intensity further demonstrated a significant increase in fluorescence signal upon treatment with targeted AgNPs.

Targeted Delivery of AgNPs to Human Glioma Tissues: Human glioma tissue slices exhibited clear fluorescence signals, confirming the binding of AgNPs within tumor tissue. Signal was heterogeneously spread over the slice with some regions showing stronger fluorescence.

5 Discussion

AgNPs have received considerable attention for cancer treatment. However, their clinical application remains limited for several reasons, mainly for their toxic effects on healthy cells. One possibility to overcome AgNPs-related obstacles is to deliver them via lipid-based drug carrier.

Our research is made of two parts. In the first one, we developed pure liposome-encapsulated AgNPs, free of unencapsulated AgNPs, and investigated their properties. It is worth noting that this work is the first to purify liposome-delivered AgNPs and evaluate their net effect.

Characterization showed that AgNPs-c were smaller due to the stabilizing role of citrate, which prevents the aggregation of AgNPs unlike AgNPs-u. This was further supported by Zeta potential data, where AgNPs-c exhibited a strongly negative Zeta potential leading to repulsion between particles.

The relatively large size of AgNPs-u makes their incorporation into a 3–4 nm thick hydrophobic bilayer core highly unlikely. In parallel, citrate-stabilized AgNPs carry a highly negative charge creating electrostatic repulsion with negatively charged lipids. Instead, both NP types were anticipated to settle within the inner core of LUVs.

To study the direct impact of LAGs, it was necessary to filter these particles from the influence of free AgNPs not present within the liposomes, and for this purpose SEC was employed. LAGs penetrated through SEC column faster, while smaller free AgNPs remained at the top of the column, likely due to strong nonspecific interactions with the stationary phase.

Zeta potential measurements revealed that encapsulation altered surface charge properties of LAG formulations. This may be attributed to a possible and partial intercalation of AgNPs into the inner surface of the bilayer and the resulting morphological distortions in the liposomal membrane.

DGC results showed that LAGs were consistently found to be denser than either free AgNPs or empty liposomes; however, a complete physical separation by DGC was not feasible proposing that DGC could only serve as a useful qualitative and semi-quantitative method for assessing liposome loading. According to the lipid content results, PC-LAGs and especially PC/PG/SM-LAGs were more enriched in denser fractions than empty liposomes, advising that PC, PG and SM enhance AgNP encapsulation and retention.

Stability assays highlight the critical role of medium composition in maintaining the integrity of LAGs. In this context, LAGs composed of PC alone and PIP₂-containing LAGs were the least stable. This is believed to be the result of PC zwitterionic charge. Meanwhile, PIP₂ strong negative charge

could cause electrostatic repulsion between adjacent PIP₂ heads leading to CF release. Conversely, PG, PG/SM, PG/SM/Cho were the most stable in such conditions. Explanations include the rigidifying effect of both SM and Cho.

Using FBS further enhanced the observed differences. For example, LAGs composed of PC alone, as well as containing both PS and PIP₂, were unstable in this medium, possibly due to an interaction between serum proteins and these PLs. This may stem from the destabilizing effect of the serum proteins. For the remaining formulations, PC/PG-, PC/PG/SM-, and PC/PG/SM/Chol-LAGs results were consistent with previous results, with these three formulations being the best candidate for bioassays.

EEs was estimated to be less than 30%, due to the relatively large size of AgNPs, in addition to the repulsion between AgNP negative charges and negatively charged PLs in the liposome bilayer. Both free AgNPs and LAGs were toxic toward A375 and RPMI7951, with A375 being more vulnerable. Data also show that encapsulating AgNPs within liposomes could improve their toxic performance toward tumor cells. While, when applied to healthy HEK293 cells, LAGs were less toxic compared to their AgNP control. This is probably due to the well-known enhanced internalization and turnover of tumor vs. normal cells.

For long-term exposure studies both AgNPs and LAGs inhibited the clonogenic potential of A375 and RPMI-7951 in a comparable way, with some formulations showing enhanced activity over their AgNP control.

Moving toward 3D models, A375 spheroid invasion was significantly suppressed by both types of AgNPs and LAGs, meaning that encapsulation preserved the antiinvasive effect of AgNPs. As for RPMI7951 results, only AgNPs-c and LAGs-c were effective with similar efficacy. These findings suggest that their efficacy is strongly influenced by NP surface chemistry and the invasive phenotype of the tumor cells. In contrast to A375 cells that show mostly ameboid type of invasion, differential sensitivity of RPMI7951 spheroids is likely due to their mesenchymal-like invasion.

Having optimized an encapsulation method, we next focused on an alternative lipid-AgNP delivery approach. By coating AgNPs with lipids, a higher rate of AgNP shielding is achievable. Despite an advantage of higher AgNP load, it is a technical challenge to prevent the lipid-coated AgNPs from degradation, so to address these technical challenges and make triangular lipid-coated AgNPs a realistic alternative we assembled stable and targeted NPs and intended to use PTT.

PTT is a medical minimally invasive approach for cancer, which exploits the ability of PTAs to induce localized hyperthermia. Our lipid-coated triangular AgNPs exhibited SPR peak within the NIR region (650-950 nm), confirming their suitability as PTAs. This was achieved by employing H₂O₂ as an oxidative etchant, thereby controlling the shape of AgNPs, and favoring the formation of triangular particles. TEM analysis demonstrated that lipid-coated AgNPs have triangular morphology, where sharp edges and tips of this morphology act as plasmonic spots tuning AgNP SPR into the biocompatible NIR region.

Vulnerability of AgNPs to oxidative dissolution and surface corrosion in aqueous and biological environments, leading to instability and enhanced Ag⁺ release is a limiting factor for their usage. To directly evaluate the effectiveness of surface coatings in protecting AgNPs from such chemical degradation, cyanide was employed to rigorously assess NP surface coverage. Exposure to KCN revealed that bare AgNPs, as well as partially functionalized formulations were highly susceptible to etching, indicating insufficient shielding of the silver core. In contrast, only AgNPs–SOA–DPPC–HT formulation exhibited pronounced resistance, demonstrating that the combined effect of SOA as a hydrophobic binding partner, DPPC as a lipid membrane, and HT as an anchoring agent all produced a dense and continuous protective coating.

Consistent with LAg results, hybrid coating also markedly reduced toxicity to HEK293 cells compared to bare AgNPs. This may be attributed to the strong shielding effect of the coat which prevents AgNP premature oxidation upon application. While for GBM tumor cells, viability test conclude that coating did not affect the cytotoxic properties. In addition, exposing cells to an external laser light after treatment with triangular AgNPs enhanced the therapeutic performance against both U87 and U251 cell lines. This is due PTT effects, that is provoking hyperthermia to levels exceeding cell tolerance, leading to irreversible cellular. In short, lipid-coated AgNPs achieved a dual effect, acting as both a tumor-directed toxic agent and a PTA.

To increase the effectiveness of these particles, we next employed active targeting by functionalizing the coating layer with different targeting molecules, including CTX, EGF, and folate. CTX is a peptide found in scorpion venom that has high affinity for GBM cells. Furthermore, approximately 40% of GBM patients exhibit EGFR overexpression, which encourages the use of EGF. Additionally, folate was also chosen due to the high levels of folate receptors in GBM. Spheroid targeting results revealed that targeted coated AgNPs significantly improved NP docking and uptake shown as an increase in fluorescence signal.

Building on the observations with 3D GBM spheroids, we next assessed the accumulation of targeted AgNPs in human glioma tissue slices to examine AgNP delivery in a complex, *ex vivo* tumor environment. A heterogeneous signal within each slice was noticed. Differences in receptor density, cell composition, and abnormal extracellular matrix within the tumor likely influence where and how CTX-, folate-, and EGF-functionalized AgNPs accumulate, highlighting the role of targeting ligands in directing targeted AgNPs to specific regions of the tumor tissue.

In summary, lipid-coated triangular AgNPs combine stability, NIR-responsive photothermal activity, and toxicity against GBM cells, while reducing toxicity toward healthy cells. Targeting ligands further enhanced tumor-specific accumulation, and PTT amplified their anticancer effects. Future studies are needed to evaluate long term therapeutic efficacy, biodistribution, and potential anti-invasive properties.

6 Conclusion

AgNPs are of great importance in the medical field due to their unique biocidal properties, but their clinical application still suffers from issues related to their stability and uncontrolled toxicity. Therefore, it appeared appealing to overcome these drawbacks by delivering AgNPs using lipid carriers, either in the form of liposomes or by coating them with a lipid bilayer.

LAg results showed that their purification using SEC allowed the study of their net effect without interference from unencapsulated AgNPs. Some LAg formulations exhibited high stability in bio media, enabling their application in cellular assays. AgNPs encapsulation reduced their off-target cytotoxicity towards healthy cells, while maintained or enhanced both short- and long-term passive efficacy against melanoma cells. Anti-invasive activity in 3D tumor models was also maintained. Lipid-coated triangular AgNPs exhibited suitable NIR optical properties and high stability against strong oxidants, along with excellent photothermal properties, making them suitable for use as a PTA. AgNPs coating reduced the uncontrolled toxicity of AgNPs while maintaining their activity against GBM cell lines. Laser application enhanced coated AgNPs effectiveness by inducing hyperthermia. Surface modulation with targeting agents improved fluorescence signal, indicating an improved uptake rate. Targeted AgNPs were able to bind to human tissue slices showing heterogenous signal distribution.

These results keep the hopes in lipid-AgNP formulations, as these lipid-based delivery methods offer the possibility of overcoming AgNPs-related limitations and improving efficacy. Future studies in complex *in vivo* models will be essential to validate the safety, biodistribution, and tumor-targeting capabilities of these formulations.

7 Publications

The thesis is based on the following publication:

Darwish, Ammar, Nikolett Sándor, Imre Szent, Tamás Marosvölgyi, Kata Juhász, Andrea Rónavári, Edi Kachal, Bence Kutus, Zoltán Kónya, and Zsolt Balogi. "Highly Stable Antitumor Silver-Lipid Nanoparticles Optimized for Targeted Therapy." **International Journal of Nanomedicine** (2025): 1351-1366. **IF: 6.5; D1**

Darwish, Ammar, Milán Pammer, Ferenc Gallyas Jr, László Vígh, Zsolt Balogi, and Kata Juhász. "Emerging lipid targets in glioblastoma." **Cancers** 16, no. 2 (2024): 397. **IF: 4.4; Q1**

8 Conference attendance

Ammar Darwish, Nikolett Sándor, Imre Szent, Tamás Marosvölgyi, Kata Juhász, Andrea Rónavári, Edi Kachal, Bence Kutus, Zoltán Kónya, Zsolt Balogi
Silver-lipid nanoparticles shaped for targeted tumor therapy
ÖPhG MEETING: Pharmaceutical Sciences in the Heart of Europe, Vienna 2025
Participation type: **Oral lecture (selected)**.

Ammar Darwish, Nikolett Sándor, Imre Szent, Tamás Marosvölgyi, Kata Juhász, Andrea Rónavári, Edi Kachal, Bence Kutus, Zoltán Kónya, Zsolt Balogi
Silver-lipid nanoparticles shaped for targeted tumor therapy
Annual Meeting of the Hungarian Biochemical Society, Budapest 2024
Participation type: **Poster (prize winner)**.