European Review for Medical and Pharmacological Sciences 2024; 28: 1417-1422

Silver nanoparticles enhanced enzyme-linked immunosorbent assay (ELISA) detection of cancer testis antigens (CTAs)

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Abstract. – OBJECTIVE: The Enzyme-Linked Immunosorbent Assay (ELISA) has been a cornerstone technique in laboratory medicine for over 55 years, relying on the specific binding of antibodies to antigens. ELISA's widespread use stems from its ability to detect low concentrations, its specificity, reproducibility, and potential for high-throughput screening. However, its sensitivity has limitations, prompting the exploration of innovative methods to improve the limit of detection (LOD). Nanoparticles provide a promising platform for enhancing ELISA sensitivity. Due to their high surface-to-volume ratio, they offer increased binding sites for capture elements and reporting tags, leading to amplified analytical signals. Recent studies have demonstrated improved sensitivity in ELISA through nanoparticle application, yielding faster detection times and enhanced sensitivities. This study investigates the potential of 50 nm citrate-capped silver nanoparticles to enhance ELISA's performance in quantifying cancer testis antigens (CTAs).

PATIENTS AND METHODS: In our study, we used the Human NY-ESO-1 ELISA kit (for research purposes) to determine the concentration of CTAs in randomly selected samples from healthy (n=89) and oncological (n=80) subjects, aged 18-75. We employed 50 nm citrate-capped silver nanoparticles (AGCB50-1M, BioPure Silver Nanoparticles – bare citrate, nano-Composix, San Diego, CA, USA). ELISA reactions followed the manufacturer's instructions, and data processing aligned with the same guidelines. Absorbance (OD) measurements occurred at 450 nm, influencing nanoparticle selection. Each ELISA well contained 5 ml of nanoparticles' stock solution with specified concentrations. CTAs concentrations were derived from the standard curve through CurveExpert Basic software. Statistical analysis was performed using SPSS v. 27 software, with p-values indicating significance if <0.03. The study adhered to Helsinki Declaration principles and received ethical approval. Participants provided informed written consent.

RESULTS: The increased concentration values of CTAs for healthy individuals and cancer patients were determined in the case of the application of silver nanoparticles.

CONCLUSIONS: The usage of nanoparticles can enhance the sensitivity of the ELISA method and positively influence its specific detection limit.

Key Words:
ELISA, Nanoparticles, Limit of detection.

Introduction

The Enzyme-linked immunosorbent assay (ELISA) is a technique that relies on the specific binding of antibodies to antigens and has a history of over 55 years[1]. The method stands as the most commonly employed one in laboratory medicine for routine analysis and monitoring[2,3]. Initially, the interaction between antigens and antibodies was tracked using radioactive elements, but these were later replaced by enzymatic systems for simpler and safer reading. Among these, peroxidase (horseradish peroxidase - HRP) has emerged as the preferred reporter enzyme due to its stability and efficacy[2,3]. ELISA's success lies in its capability to detect low concentrations, its specificity, reproducibility, and its potential for high throughput screening, although it typically requires several hours to yield results[3].

Despite its numerous advantages, ELISA's sensitivity is constrained in certain scenarios[4], prompting the exploration of innovative approaches to enhance its limit of detection (LOD). Strategies to increase sensitivity include the application of redox complexes, electroactive molecules, and metal ions[5,6]. In this pursuit, nanotechnology-based strategies featuring nanoparticle solutions have gained traction[4-12].

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Nanoparticles offer a superb platform for carrying specific recognition molecules like antibodies, probes, and reporter molecules. Their high surface-to-volume ratio provides more binding sites for capture elements and reporting tags, leading to an amplified analytical signal within a single recognition event[5,7]. Our team reported the improvement of ELISA method sensitivity by application of nanoparticles[8]. Luo et al[13] demonstrated improved sensitivities and faster detection times for C-reactive proteins through a quantum-dot-labeled immunoassay. Similarly, Zhang et al[6] observed a 5,000-fold sensitivity enhancement for detecting the ataxia telangiectasia mutated protein using functionalized multi-walled carbon nanotubes.

The most important aspect in the case of nanoparticles is the attachment of biomolecules to them. This process can alter the nanoparticles' properties and biochemical activity. Factors such as surface chemistry, pH, stabilizing agents, and the addition process significantly influence the final coverage and efficiency of biomolecules[14,15]. Additionally, the binding of biomolecules to nanoparticles can be accomplished through various methods. Biomolecules can be adsorbed onto the nanoparticle surface through electrostatic or hydrophobic interactions, resulting in a high number of proteins per particle and a random orientation[9,12]. Conversely, more stable covalent immobilization has been reported in other studies[16-18], offering better control over particle coverage and even binding orientation. Each of these methods has its advantages and drawbacks, such as potential leakage of non-covalently attached biomolecules or loss of biomolecule activity due to aggressive protocols[19,20]. Therefore, the ideal conjugation strategy depends on the intended application. Notably, there is a lack of specific studies on how different conjugation strategies affect the potential of nanocomplexes to enhance ELISA sensitivity.

As a result, we decided to test whether 50 nm citrate-capped silver nanoparticles could improve ELISA performance for a quantitative determination of concrete biomarkers – cancer testis antigens (CTAs). These proteins are expressed in different tumors and at concrete stages of spermatogenesis; their aberrant expression in cancer, as well as the correlation of CTAs expression with chromosomal instability and structural chromosomal abnormalities, has been reported[21]. CTAs contribute to the regulation of different cellular processes, although their biology and expression profiles and role in cellular process regulation need further investigation. Considering the perspective role of CTAs, the elaboration of easy, non-expensive, and sensitive methods of CTAs' quantitative determination is of great diagnostic importance and value.

Patients and Methods

In our study performed in August 2023, we utilized Human NY-ESO-1 (Cancer testis antigens) ELISA kit EH10736 (Fine Biotech Co. Ltd, Wuhan, Hubei, China) intended for research purposes. The silver nanoparticles AGCB50-1M (nano-Composix, San Diego, CA, USA) employed were 50 nm in diameter and citrate capped.

The ELISA reactions were conducted in accordance with the manufacturer's guidelines, and the resulting data were processed accordingly. To evaluate the ELISA outcomes, the absorbance (optical density - OD) was measured at a wavelength of 450 nm, a criterion that influenced the selection and suitability of the nanoparticles. Within each well of the ELISA microplates, 5 ml of the nanoparticles' stock solution was introduced [with a mass concentration of 1.0 mg/mL, an atomic (Ag) molarity of 9.27 mmol/L, a molarity particle concentration of 1.5×10^{12} particles/mL, an Ag mass percent (%) of 0.1, and a maximum optical density (cm^{-1}) of 120]. The parameters specific to the selected ELISA kit are: range 0.156-10 ng/ml, and 0.094 ng/ml sensitivity.

CTAs concentrations per sample were interpolated from the standard curve using the recommended professional software CurveExpert Basic (Hyams Development, available at: https://www.curveexpert.net) as specified by the manufacturer.

For this study, samples of a healthy population (89 donors[22], 28 females and 61 males) and oncology patients (80, 54 females and 26 males) were selected randomly. All samples were collected during the period from June 1, 2022, until March 1, 2023. All collected samples were analyzed using both a standard ELISA method and an ELISA method enhanced at the detection stage by silver nanoparticles. All subjects were 18-75 years old. In the case of oncology patients, all of them were diagnosed with breast and colorectal cancer and prostate adenocarcinoma in 2020. The samples of healthy population and oncology patients were acquired as part of our team's research activities and approved by the Bioethics International Committee of the Petre Shotadze Tbilisi Medical

Table I. Cancer testis antigens (CTAs) expression/concentration determined without [CTA (ng/ml)] and with [CTA (ng/ml), Ag] usage of silver nanoparticles (Ag) across disease status and sex.

		Female	Male	Total
CTA (ng/ml)	Cancer	0.63 ± 0.3	0.64 ± 0.3	0.56 ± 0.3
	Healthy	0.34 ± 0.26	0.37 ± 0.26	0.3 ± 0.26
CTA (ng/ml), Ag	Cancer	0.78 ± 0.41	0.87 ± 0.39	0.81 ± 0.15
	Healthy	0.47 ± 0.19	0.48 ± 0.2	0.42 ± 0.12
n	Cancer	54	26	80
	Healthy	28	61	89
Mean Age	Cancer	50.2	72.5	56.0
	Healthy	38.0	36.6	37.0

Academy (IRB45765021/9). All procedures adhered to the principles of the Helsinki Declaration (revised in 2013). Participants were informed about the study, and their written informed consent for inclusion and anonymous data publication was obtained prior to their participation.

Statistical Analysis

Statistical analysis of the results was carried out using SPSS 27 (IBM Corp., Armonk, NY, USA). The approach of descriptive statistics was applied to determine the main features of the data collected from both the conventional ELISA test and the modified ELISA test with silver nanoparticles. This includes measures of central tendency - mean and measures of dispersion. To ascertain the significance of the results, the *p*-value was considered significant if <0.03.

Results

To test the hypothesis that ELISA, when enhanced with silver nanoparticles, could detect lower concentrations of CTAs, a comparative analysis was conducted between a conventional ELISA test and the same test modified with the addition of a nanoparticle stock solution. The nanoparticle stock solution comprised a mass concentration of 1.0 mg/mL, an atomic (Ag) molarity of 9.27 mmol/L, a molarity particle concentration of 1.5×10^{12} particles/mL, an Ag mass percent (%) of 0.1, and a maximum optical density (cm^{-1}) of 120. The addition of nanoparticles was executed 5 minutes prior to the introduction of the 3',3',5',5'-tetramethylbenzodine (TMB) substrate solution during the detection stage. The results of the comparative analysis revealed that the modified ELISA test with the inclusion of silver nanoparticles exhibited heightened sensitivity in detecting CTAs. This enhancement was evidenced by the increased expression/concentration values observed compared to the conventional ELISA procedure.

The expression levels of CTAs determined by ELISA without and with the application of silver nanoparticles are presented in Table I and Table II. The increased concentration values of the targeted biomarker were determined for healthy populations and cancer patients in the case of the application of silver nanoparticles.

Table II. Cancer testis antigens (CTAs) expression/concentration determined without [CTA (ng/ml)] and with [(CTA (ng/ml), Ag)] usage of silver nanoparticles (Ag) across disease status, oncology diagnosis, and sex.

Diagnosis	Biomarker	Mean	Lower 95%	Upper 95%	Unit
Breast cancer	CTA	0.633	0.532	0.735	ng/ml
Mean age = 49.8, n = 51	CTA-Ag	0.792	0.703	0.954	ng/ml
Prostate adnocarcinoma	CTA	0.683	0.533	0.834	ng/ml
Mean age = 74.7, n = 22	CTA-Ag	0.866	0.503	1.185	ng/ml
Colorectal cancer	CTA	0.513	0.354	0.673	ng/ml
Mean age = 59.0, n = 7	CTA-Ag	0.801	0.798	2.446	ng/ml
Healthy Female	CTA	0.344	0.221	0.467	ng/ml
Mean age = 38.0, n = 28	CTA-Ag	0.401	0.128	0.447	ng/ml
Healthy Male	CTA	0.367	0.292	0.443	ng/ml
Mean age = 36.6, n = 61	CTA-Ag	0.432	0.131	0.458	ng/ml

Discussion

We anticipated that ELISA, when enhanced with silver nanoparticles, would be capable of detecting lower concentrations of target biomarkers (CTAs). To validate this statement, we conducted a comparative analysis between a conventional ELISA test and the same test modified with the addition of a nanoparticle stock solution [with a mass concentration of 1.0 mg/mL, an atomic (Ag) molarity of 9.27 mmol/L, a molarity particle concentration of 1.5×10^{12} particles/mL, an Ag mass percent (%) of 0.1, and a maximum optical density (cm^{-1}) of 120] during the detection stage. The introduction of nanoparticles was performed 5 minutes prior to the addition of the 3',3',5',5'-tetramethylbenzodine (TMB) substrate solution.

Our findings indicated increased expression/concentration values of CTAs when the ELISA procedure was enhanced with the application of nanoparticles. However, we did not perform any modification of nanoparticles that may ensure the physical and chemical interactions that occur between nanoparticles and targeted proteins[23]. These possibilities should be investigated additionally.

Our study suggests that the use of silver nanoparticles could potentially improve the sensitivity of the ELISA test, allowing for the detection of lower concentrations of CTAs. Nanoparticles possess distinctive characteristics, including stability and customizable optical properties, rendering them highly effective for biosensing applications. As an illustration, metal nanoclusters (NCs) have been employed to identify biomarkers associated with diverse diseases, encompassing infectious, inflammatory, or neoplastic conditions[24]. Furthermore, biosensors incorporating nanotechnology demonstrate exceptional sensitivity, specificity, and the ability to recognize multiple molecules, making them well-suited for the identification of extracellular cancer biomarkers such as cancer-associated proteins, circulating tumor DNA, and extracellular vesicles[25]. Additionally, plasmonic nanoparticles have found application in *in-vitro* sensing methodologies like Surface-enhanced Raman scattering (SERS)[26], Surface-enhanced fluorescence (SEF), colorimetric assays, and Localized Surface Plasmon Resonance (LSPR), enhancing the sensitivity of detecting key biomarkers linked to Alzheimer's Disease in bodily fluids[27].

Limitations

However, there are several limitations to our study that should be considered:

1. Lack of nanoparticle modification: In our study, we did not perform any modification of the nanoparticles that may ensure the physical and chemical interactions that occur between nanoparticles and targeted proteins. The impact of such modifications on the sensitivity of the ELISA test is unknown and warrants further investigation.
2. Timing of nanoparticle introduction: The introduction of nanoparticles was performed 5 minutes prior to the addition of the 3',3',5',5'-tetramethylbenzodine (TMB) substrate solution. The optimal timing for the introduction of nanoparticles to maximize the sensitivity of the ELISA test is not known and should be explored in future studies.
3. Nanoparticle concentration: The nanoparticle stock solution used in our study had a mass concentration of 1.0 mg/mL, an atomic (Ag) molarity of 9.27 mmol/L, a molarity particle concentration of 1.5×1,012 particles/mL, an Ag mass percent (%) of 0.1, and a maximum optical density (cm^{-1}) of 120. The effect of varying these parameters on the sensitivity of the ELISA test is not known and should be investigated.

Thus, while our study provides preliminary evidence supporting the use of silver nanoparticles to enhance the sensitivity of the ELISA test for the detection of CTAs, further research is needed to optimize the procedure and fully understand the underlying mechanisms. The use of nanoparticles in the detection of biomarkers holds great promise for early disease detection and diagnosis[28].

Conclusions

The usage of nanoparticles can enhance the sensitivity of the ELISA method and positively influence its specific detection limit. We assume it is logical and effective to augment the sensitivity of the ELISA method through nanoparticle incorporation. This approach is cost-effective and will positively contribute to the method's accuracy.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements
The authors acknowledge Jo Ann Medical Centre's Blood Bank and Tbilisi Cancer Center for their support in the process of sample collection. .

Ethics Approval
The present study has been approved by the Bioethics International Committee of the Petre Shotadze Tbilisi Medical Academy (IRB45765021/9). All procedures performed in the present study were in accordance with the Helsinki Declaration (as revised in 2013). The samples used in the present study were collected from the registered participants.

Informed Consent
The participants were informed about the study design and objectives. All participants provided informed consent for inclusion and for anonymous data publication before they participated in the study. The information about the study, including the study design and objectives, was presented to the potential participants together with the registration form.

Data Availability
The datasets created and analyzed during the current study are available from the corresponding author upon reasonable request.

Funding
The publication is prepared in the frames of the project "Cancer testis antigens as the early biomarkers of oncogenesis"; this study was supported in the frames of Petre Shotadze Tbilisi Medical Academy funding program "Facilitation of scientific-research activities".

Authors' Contribution
All authors contributed equally to this article.

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