Tikrit Journal of Veterinary Sciences (2025) 4(1): 74-83
DOI : 10.25130/tjvs.4.1.10

IRAQI Academic Scientific Journals

TJVS Tikrit Journal of Veterinary Sciences

Tikrit Journal of Veterinary Science

Detection of Hemolytic Activity of Brucella melitensis Antigens Loaded in Nanocarriers

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ARTICLE INFO.

Article history:
-Received: 21/11/2024
- Received In Revised Form: 2/1/2025
-Accepted: 14/ 1/ 2025
-Available online: 30/ 6/2025

Keywords:
***Brucella melitensis* Antigens, Ca, P/Cs NPs, hemolysis assays, hemolysis index.**

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ABSTRACT

The hemolytic potential of NPs has been the subject of numerous studies as a critical indicator of their safety in vaccine applications. In this study, the elemental compositions of Brucella melitensis Antigens (BMAs) loaded in Ca, Phosphate/ Chitosan nanocomposite created by encapsulation method. In addition, size, and surface topography were characterized by UV-VIS spectrophotometers, Energy Dispersive X-ray (EDS/EDX), and Atomic Force Microscopy (AFM) techniques. Furthermore, the cytotoxicity of Brucella melitensis antigens loaded in calcium phosphate/chitosan nanoparticles was determined by measuring the hemoglobin released from rabbit erythrocytes using a spectrophotometer at 545 nm. The results showed that λ max of BMAs, Ca, P/CsNPs were present at 1.987 at the wavelength 232 nm which differed from the absorbance values of CaP/Cs NPs and Brucella melitensis Antigens samples respectively. The main components of BMAs, Ca, P/CsNPs were C, O, and sodium, with 22.2%, 46.1%, and 20.7 % respectively and the secondary components were Ca and P with 4.4% and 1.3 % respectively. Additionally, other elements confirmed the presence of Brucella melitensis antigens in. nanocomposite. The average particle size was 30.15 nm and a total height of 107.4 nm. Additionally, the hemolysis index of BMAs, Ca, P/Cs NPs was not detectable against rabbit RBCs. We can conclude that the unique properties of CaP/CsNPs enabled the carry of Brucella melitensis antigens and were nontoxic for RBCs, suggesting potential candidates for vaccine applications against Brucella melitensis.

Introduction

The brucellosis disease significantly impacts global trade, costing many countries between $30 and $300 million in lost revenue (1). The disease causes abortion or infertility in livestock, leading to significant persistent infections that require expensive and long-term antibiotic treatment (2). Gram-negative bacteria of the genus Brucella, particularly Brucella melitensis, Brucella abortus, and Brucella suis, are the most virulent in humans and cause brucellosis, which the World Health Organization has identified as one of the most "neglected zoonoses"(3). Cattle, sheep, and other livestock must be immunized and killed to prevent brucellosis. These are still challenges, however, particularly in distinguishing between animals that were given vaccinations and those that are naturally infected (4).In addition, euthanasia may be more difficult to differentiate between vaccine-induced immunity and spontaneous infection if vaccination proves unsuccessful and produces abortion in pregnant animals (1). There are three main live attenuated vaccines for livestock S19 and RB51 for cattle, and Rev1 for small ruminants they control brucellosis but are not perfect; they can be pathogenic in humans and have residual toxicity in animals while not providing complete protection against virulent strains (5).

A nanoparticle is defined as a particle with at least one dimension of <100 nm and these molecules can function as both a delivery system and an immune-stimulating adjuvant(6). Chitosan and CaPNPs have unique properties including biodegradability, biocompatibility, nontoxicity, and low cost, and used as the delivery vehicle for various biomolecules including peptides, proteins, antigens, genes, and oligonucleotides (7 - 8) The combination of Calcium phosphate and chitosan nanoparticles (CaPNPs) as adjuvants is promising for vaccine development and usage in medical applications (9- 10) However, the limitations of NPs for the delivery of vaccines range from concerns over the toxicity of the particles to difficulties in producing the materials and presenting antigens in their native form(7)(11).). The rapid advancement of nanomedicine and the increasing development of new nanoparticles (NPs) have made in vivo toxicity testing both ethically and financially undesirable. (12). Among various cell types, red blood cells (RBCs) are particularly useful for toxicity assessments because NPs readily enter the bloodstream and interact with RBCs, which are abundant and well-characterized(13). This study aims to confirm the properties of Brucella melitensis Antigens loaded in Ca, Phosphate/ Chitosan nanoparticles (BMAs, Ca, P/ Cs NPs) by UV, EDX, and AFM analysis techniques and detect its hemolytic activity using Rabbit RBCs.

Material and methods

Formulation of Brucella melitensis Antigens Ca, Phosphate/ Chitosan Nanoparticles

Whole-cell Antigens of Brucella melitensis (BMAs) were prepared by sonication process at the Veterinary Medicine Microbiology lab at Tikrit University; conducted according to Mitov et al., (1992) (14). Nanoencapsulation of the B.melitensis Antigens suspension was prepared following Abkar et al., (2019) and Pei et al., (2019)(15-16) methods with slight modification by combining Ca, Phosphate/Chitosan nanoparticles with B.melitensis Antigens and adding acidified TPP solution. The preparation and confirmation measurements of nanoparticles were applied at the Ministry of Science and Technology, Iraq, and at the University of Kashan, Iran. The concentration of free protein in BMAs, Ca, P/Cs nanoparticle suspension was determined using a Bradford protein assay(17).

Nano properties BM antigens loaded in Ca, P/CsNPs

Absorption spectra, elemental and topography of BM antigens loaded in Ca, P/Cs Nanoparticles were analyzed by UV-VIS spectrophotometer (UV-VIS), Energy Dispersive X-ray(EDX), and Atomic Force Microscopy (AFM) and compared with BM antigens and Ca, P/Cs Nanoparticles

UV-VIS

Dual-beam UV-VIS spectrophotometers were used to measure the absorption spectra of the BMAs,. Ca, P/CsNPs- solution according to the method of Agarwal et al., (2018)(18). All spectra were taken in a 1 cm optical path quartz cell at room temperature with distilled deionized water as a control. The absorbance was calculated at (200-800nm), and the concentrated samples were diluted 1:10.

EDX

Energy Dispersive X-ray (EDS/EDX) is a significant instrument for detecting elements in Ca, P/Cs nanoparticles, and BMAS, Ca, P/Cs nanoparticles to improve the therapeutic performance of some chemotherapeutic agents.

AFM

The surface morphologies BMAs, Ca, P/ Cs NPs were observed using an Atomic Force Microscope (AFM) in contact mode under ambient air conditions. This analysis was applied using a 1x2 cm glass slide coated with drops of the nanoparticle solution, left to dry, and then distributed on the AFM sample stage to examine (19) .

Hemolytic activity of BMAs, Ca, P/ Cs NPs

The cytotoxicity for BMAs, CaP/CsNPs was determined by measuring and viewing the hemoglobin released from Rabbit erythrocytes by both hemolytic activity in tubes as well as the spectrophotometric reading (20-21) The assay was performed using Rabbit RBC 1.5ml Eppendorf tubes. The Heparinated Rabbit blood sample was packed by centrifugation at 1000g for 10 min, washed three times with PBS, and then diluted in phosphate-buffered saline (PBS, Sigma) to 2% (v/v). Two-fold serial dilution of BMAs, CaP/CsNPs was performed using PBS. Aliquots of 100 µL of each dilution were added to 100 µL of 1% erythrocyte suspension and incubated for 60 min at 37oC. For negative and positive controls 22µl of PBS and Distal water were used in place of the test sample. Then, after incubation, all samples in Eppendorf microtubes were centrifuged at 1000 g for 5 min at room temperature, and 200µl of the supernatant was diluted with 800µl PBS before measuring the absorbance at 545 nm. The supernatant from the negative control served as blank. Percentage hemolysis was deduced at the absorbance (A) ratio between each sample and the positive control(22)

$$Ha=(N\_v-NC)\div(PC-NC)\times100$$

Ha: hemoglobulin percent; Nv: Cs/CaPNPs-BME Vaccine; NC: negative control; PC: positive control.

Additionally, Microsoft EXCEL 2019 was used for analysis of data generated form hemolysis assay.

Results and discussion

The formulation of Brucella melitensis antigens (BMAs) loaded in calcium phosphate and chitosan nanocomposites was conducted for the first time, necessitating additional analyses to confirm the nanostructure of this formulation. In addition to FTIR, X-ray diffraction, and scanning electron microscopy (SEM), these techniques demonstrated the homogenized, spherical shape and high loading capacity of B. melitensis antigens within the calcium phosphate/chitosan nanoparticles.

Nanoparticles analysis

Ultraviolet spectroscopy is a preliminary spectroscopy technique used to identify the changes in the absorption peaks that occur due to changes in molecular structure (23). In the current study "λ max" (lambda max) as shown in Table 1 and Fig. 1 for B. melitensis Antigens (BMAs) was (1.558 and 0.22) at wavelengths (263 and 213) nm respectively. Furthermore, Ca, P/ CsNPs were reached (0.214; 0.207; 0.221, and 0.911) at wavelengths (793;762;752, and 236) nm respectively. while the λ max of B. m.A, Ca, P/CsNPs was (1.987) at the wavelength (232) nm. The variation in the absorbance values and wavelengths among the three samples indicates the formation of the nanomaterial and the successful loading of the BMAs into Ca, P/ Cs NPs Fig (2). The difference in Ca,P/Cs NPs, and the B. m. Antigens, Ca, P/ Cs NPs wavelengths indicated the efficiency of the synthesis process.

Table (1): UV-visible spectroscopy results of B. meltiness. Antigens; Ca, P/Cs NPs ; BMAs, Ca, P./ Cs NPs.

	No.	Wave Length	(Abs) λ max		NO.	Wave Length	(Abs) λ max
B. meltiness. **Antigens**	1	263.00	1.558	Ca, P/CsNPs	4	793	0.214
	2	213	0.22		5	762	0.207
					6	752	0.221
					7	236	0.911
BMAs,Ca, P./Cs NPs			**NO.**	**Wavelength**	**(Abs) λ max**		
			3	**232**	**1.987**		

Ab : Absorption ;CaP: Calcium phosphate ; Cs :Chitosan ;BMAs :Brucella melitensis Antigens ;NPs :Nanoparticles

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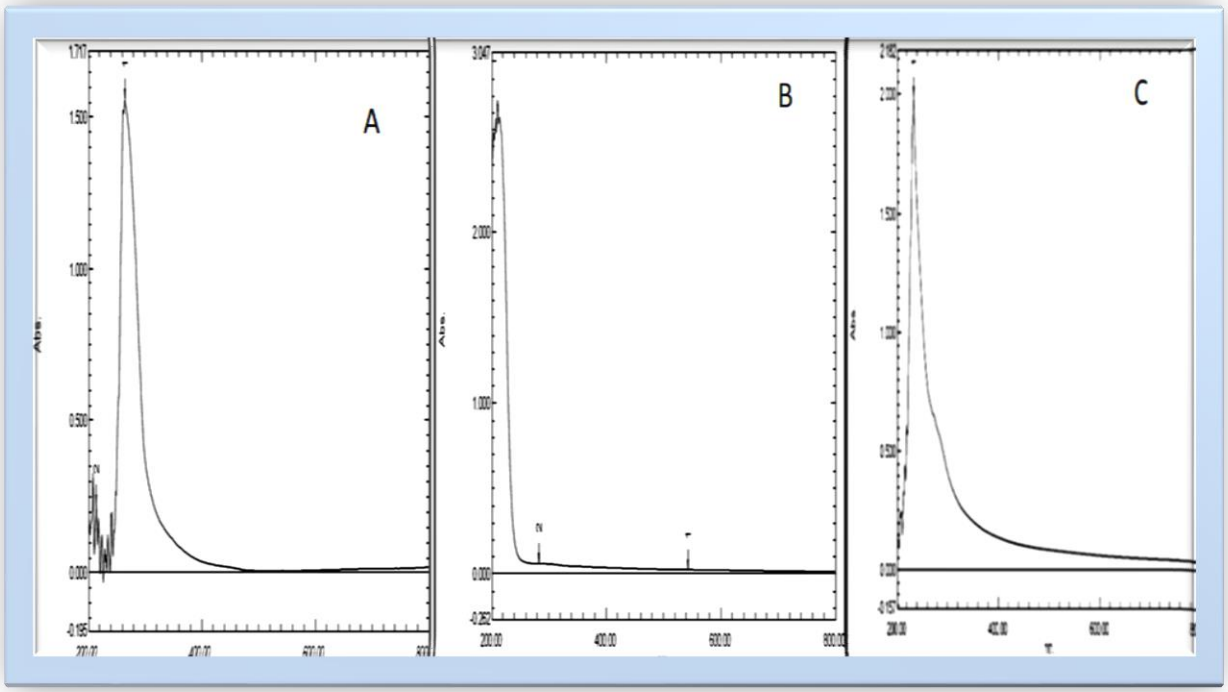

Figure (1): UV-visible spectroscopy results of A) *B. meltiness* Ex. B) Ca, P./ Cs NPs C) B. m. E. Ca, P./Cs NPs.

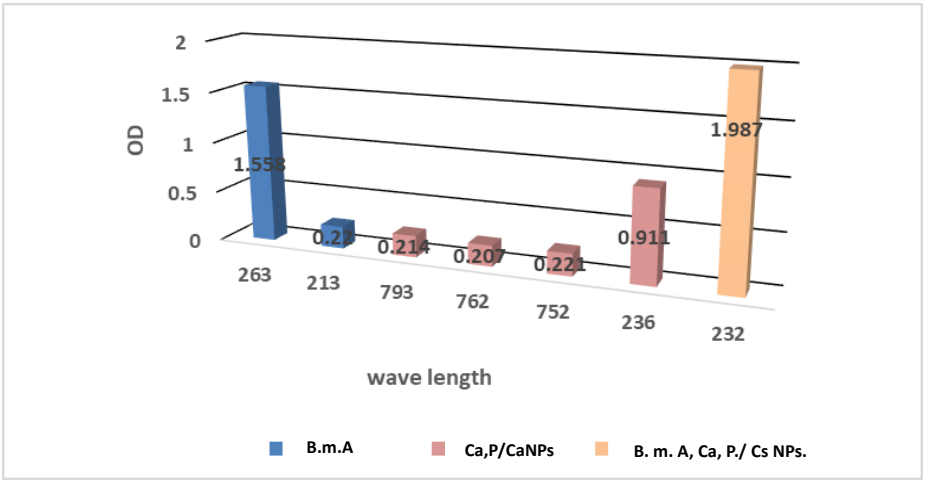

Figure (2): Diagram of UV-visible spectroscopy results of *B. meltiness* Antigens, Ca, P./ Cs NPs, and BMAs, Ca, P/ Cs NPs.

Energy Dispersive X-ray (EDX)

The elemental analysis of Calcium phosphate/chitosan nanoparticles (Ca, P/ Cs NPs), and B. meltiness Antigens loaded in Ca, P./ Cs NPs (BMAs, Ca, P./ Cs NPs) was conducted using Energy Dispersive X-ray. As shown in Table 2 and Fig.3the percentages of the main components in Ca and P/Cs nanoparticles were as follows: carbon (C) and oxygen (O) were present at 12.7% and 41.55%, respectively, derived from chitosan. The percentages oF calcium (Ca) and phosphorus (P) were 8.36% and 37.39% respectively, Furthermore, the percentages of the main components in BMAs, Ca, P/ Cs NPs as shown in Table 3 and Fig.4 were carbon(C), oxygen (O), and sodium(Na) were present at 22.2%, 46.1%, and 20.7 % respectively. The presence of secondary components such as S, Ca, P, and Cl was recorded at 4.4%, 1.7%, and 1.0 % respectively, confirming the presence of calcium and phosphate salts. Additionally, the lower concentrations of K, Mg, and Al confirmed the presence of the bacterial extract.

Table (2): EDX analysis for Elemental component of Ca, P/ CsNPs.*

No.	Element	Weight %
1	C	12.7
2	O	41.55
3	Ca	8.36
4	P	37.39

* CaP: Calcium phosphate ; Cs :Chitosan ;NPs :Nanoparticles

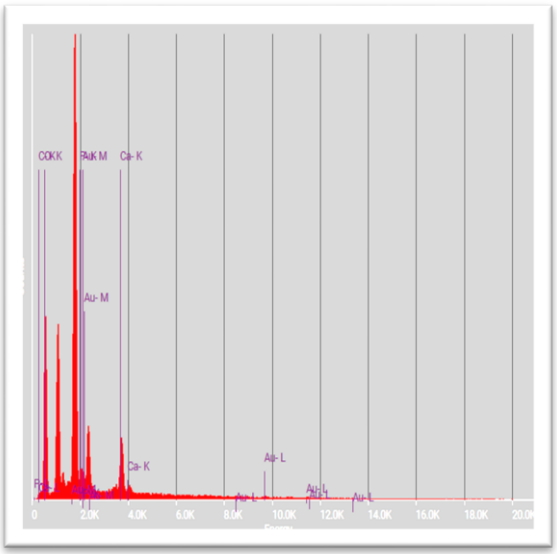

Figure (3): EDX diagram for Elemental component test of Ca, P/ CsNPs.

Table (3): EDX analysis for Elemental component of BMAs, Ca, P/ CsNPs*.

No.	Element	Weight %
1	C	22.2
2	O	46.1
3	Na	20.7
4	S	4.4
5	Si	1.3
6	Ca	1.7
7	Cl	1.0
8	P	1.0
9	Zn	0.5
10	Mg	0.3
11	Al	0.3
12	K	0.3

*BMAs :Brucella melitensis Antigens; CaP: Calcium phosphate ; Cs :Chitosan;NPs :Nanoparticles

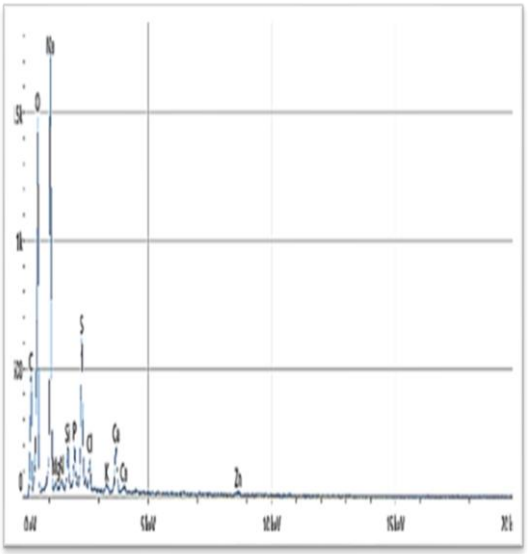

Figure (4): EDX diagram for Elemental component test of B. m. E. Ca, P/ CsNPs

Omidi and Kakanejadifard (2019) (24) argued that chitosan has four types of elements in its energy-dispersive X-ray spectroscopy (EDX) spectrum: carbon, oxygen, nitrogen, and sulfur. One possible explanation for the disappearance of nitrogen apparent in this study is that the EDX equipment was unable to detect its concentration in the sample. Electron microscopy (EDX) is typically better at detecting heavier components. When other elements are present, it could be difficult to detect nitrogen, particularly if the energy peaks of the elements overlap. Electron microscopy with Energy Dispersive X-ray Spectroscopy (EDX) is a powerful tool for elemental analysis due to their stronger X-ray emissions especially those with atomic numbers higher than 10. In, but it has limitations when it comes to detecting lighter elements such as nitrogen. Because of its atomic number of 7, produces weaker X-ray signals, making it difficult to distinguish from background noise, especially in samples containing heavier elements.(25- 26).

Atomic Force Microscopy (AFM)

In tribology, there is a distinction between smooth and rough surfaces; rough surfaces typically exhibit higher wear rates and coefficients of friction due to their ability to cause cracks or corrosion however, roughness enhances adhesion and interactions with biological systems, such as bacterial cell membranes(27)(28). the AFM has emerged as a powerful tool to obtain nano-structural details and biomechanical properties of biological samples(29). An Atomic Force

Microscopy (AFM) analysis (Fig.5) of biosynthesized calcium phosphate/chitosan nanoparticles (Ca, P/CsNPs) revealed significant shape variations characterized by sharp protrusions that create a rough texture, with a total height of 107.4 nm and an average height of 28.5 nm, enhancing their ability to penetrate bacterial cells. The observed irregular forms likely account for the wide width measurement range of 1.56 nm to 2.98 nm. These nanoparticles showed an average size of 30.15 nm with dimensions ranging from 2.03 nm to 119 nm, indicating that the biosynthetic process significantly influences their morphology and size, consistent with findings of Rasheed et al., (2017) (30).This structural complexity enhances the nanoparticles' functionality in applications such as drug delivery and antimicrobial therapies.

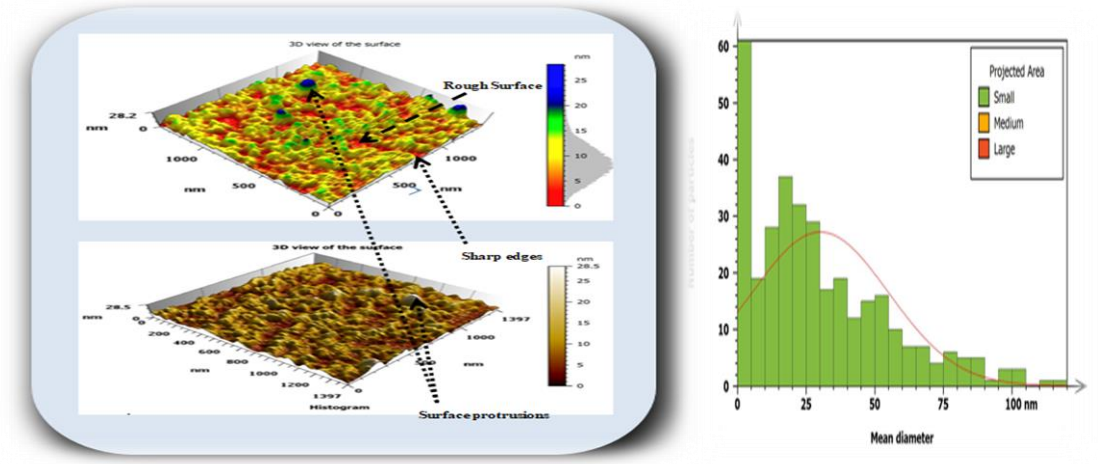

Figure (5): Three-dimensional image of B. m. E., Ca, P/ Cs NPs revealed a population of homogeneous particles with a regular surface shape in meane of size30.15 nm.

Chitosan-based NPs have very wide-ranging sizes, from 32.7 to 1100 ± 20 nm depending on the preparation methods and the targeted application (31). In this study, the average size of BMAs, CaP, and CsNPs was 30.15 nm; it was within the range to be taken up by cells. According to Tahara et al., (2009) (32) study, the size of NPs must be in the submicron range to be taken up effectively.

Hemolytic activity of nanoparticles (BMAs, Ca, P/CsNPs)

The hemolytic activities of the BMAs, Ca, P/Cs NPs against Rabbit RBCs as shown in Fig.6 were investigated to determine the toxicity on higher eukaryotic cells. The stock solution of the BMAs, Ca, P/Cs NPs with a concentration of 6 mg/ml has a slight hemolysis activity (3%). However, for the later dilutions of BMAs, Ca, P/Cs NPs showed nonhemolytic activities (0.3, 0.6, and 0%, respectively) when compared with negative and positive control samples. This test determines the percentage of hemoglobin (Hb) released after the interaction between nanoparticles (NPs) and red blood cells (RBCs).

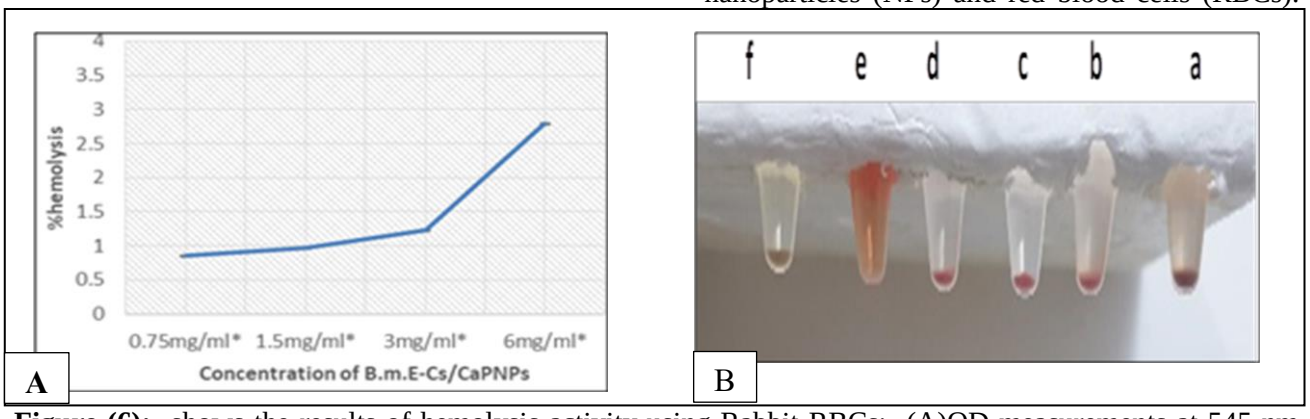

Figure (6): shows the results of hemolysis activity using Rabbit RBCs: (A)OD measurements at 545 nm (Y-axis) of free hemoglobin in 1% erythrocyte solutions originating from Rabbit, incubated for 60 min at 37 °C with PBS (negative control), D.W (positive control) and different concentrations of BMAs, BMAs, CaP/CsNPs Average values from two technical replicates are presented with error bars (SD) included in plots. B) RBCs Hemolysis testing for four concentrations of BMAs, CaP/CsNPs in tubes: (a) (6mg/ml), (b) (3mg/ml), (c) (1.5mg/ml), (d) (0.75mg/ml), (e) Positive Control (PC) and (F) PBS-Negative Control (NC).

The hemolytic assay has shown to be a promising method for evaluating the toxicity of nanomaterials because it's inexpensive, reproducible, and provides quick results. So far, hemolytic activity has even been observed with therapeutic nanoparticles both in lab settings and in living organisms, suggesting that NPs might have potentially negative effects that could restrict their use in nanomedicine (12). In this study the initial concentration of BM.A, CaP/CsNPs (6 mg/ml) was classified as nonhemolytic nanoparticles According to the hemolysis index of the American Society for Testing and Materials Designation ASTM guidelines, nanoparticles are classified as non-hemolytic if they produce a hemolysis rate of 5% or lower.

The hemolytic potential of NPs has been the subject of numerous research as a crucial safety indicator; nevertheless, since methods for testing differ, and may be challenging to evaluate information from various investigations (21). The American Society for Testing and Materials (ASTM) established a standard method to evaluate the hemolytic properties of NPs by measuring the amount of hemoglobin released during NP-RBC interaction. considering its low cost, reliability, and fast results, this hemolytic assay is a possible method to measure the toxicity of nanomaterials (33 (34)BMAs, Ca, and P/Cs nanoparticles (NPs) are nonhemolytic towards rabbit red blood cells (RBCs). This is attributed to the formulation process of the NPs, which includes tripolyphosphate (TPP). According to the findings of Albalawi et al., (2023) (35), TPP-chitosan nanoparticles demonstrate low cytotoxicity towards normal cells, indicating their potential as safer options for drug delivery. Additionally, calcium phosphate (CaP) is a naturally occurring component in the human body. CaP NPs are reported to be biocompatible, bioresorbable, and safe, with no side effects noted, as confirmed by FDA approval (10). Chitosan nanoparticles have become of great interest as polymeric platforms for the development of new pharmacological and therapeutic drug release systems with improved biodistribution increased specificity and sensitivity, and reduced pharmacological toxicity. chitosan nanoparticles have shown adjuvant effects in vaccines. Absorption and bioavailability of drugs encapsulated into chitosan nanoparticles can be improved, so they can be used to deliver gene drugs, protein drugs, and other compounds and can protect them effectively from enzyme degradation in vivo(36).

Conclusion

In this study, the size, morphology, and nonhemolytic activity of Brucella melitensis antigen-loaded in TPP-chitosan/CaP nanoparticles is a promising candidate for biomedical applications.

Acknowledgment

The authors would like to express their gratitude to Dr. Labib Ahmed Kadhim Al-Zubaidi / Senior Scientific Researcher at the Ministry of Science and Technology, Iraq, for his valuable support and guidance throughout our study.

Conflict of Interest

The authors declare there is no conflict of interest

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Tikrit Journal of Veterinary Sciences (2025) 4(1): 74-83
DOI : 10.25130/tjvs.4.1.10

الكشف عن النشاط الانحلالي الدموي لمستضدات Brucella melitensis المحملة على حاملات نانوية

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فرع الأحياء المجهرية، كلية الطب البيطري، جامعة تكريت، تكريت، العراق

الملخص

يمثل فحص قدرة الجسيمات النانوية على تحليل كريات الدم الحمراء من الفحوصات المهمة باعتبارها مؤشرا حاسما على سلامة استخدامها في تطبيقات اللقاحات.. في هذه الدراسة، تم تحديد العناصر المكونة لتوليفة مستضدات البروسيلا ماليتنس Brucella melitensis (BMAs) المحملة على جسيمات الكالسيوم، والفوسفات/ الكيتوسان النانوية بطريقة التغليف النانوي وقياس حجم، وتضاريس السطح للجسيمات النانوية بواسطة أجهزة قياس الطيف المرئي-UV VISوالأشعة السينية المشتتة للطاقة (EDS/EDX) ، والمجهر الذري للقوةAFM .وعلاوة على ذلك، تم تحديد السمية الخلوية لها عن طريق قياس الهيموجلوبين المتحرر من كريات الدم الحمراء للأرانب على طول موجي 545 نانوميتر.

أظهرت النتائج أن λ max لمستضدات والكالسيوم، وفوسفات الكالسيوم/ الكيتوسان النانوية كانت ظاهرة عند 1.987 عند الطول الموجي 232 نانومتر والتي تختلف عن قيم الامتصاص لمستضدات (Brucella melitensis (BMAs وجسيمات فوسفات الكالسيوم النانوية على التوالي. وأيضا أظهرت التحاليل أن المكونات الرئيسية لـها كالاتي الكاربون C والأوكسجين O والصوديومNa بنسب 22.2٪ و46.1٪ و20.7٪ على التوالي وكانت المكونات الثانوية هي الكالسيوم Ca والفسفور P بنسبة (4.4 و1.3) ٪ على التوالي. بالإضافة إلى ذلك الى وجود عناصر أخرى تعود الى مستضدات Brucella melitensis، أما متوسط حجم توليفة مستضدات المحملة على جسيمات النانوية كانت بقياس 30.15 نانومتر وارتفاع إجمالي 107.4 نانومتر، لم يكن مؤشر انحلال الدم لـ BMAs المحملة على جزيئات فوسفات الكالسيوم والكيتوسان النانوية قابلاً للكشف ضد خلايا الدم الحمراء للأرانب. يمكننا أن نستنتج الخصائص النانوية المميزة لتوليفة مستضدات البروسيلا مع الكالسيوم والكيتوسان وعدم سميتها لكريات الدم الحمراء يمكن استخدامها في تحضير اللقاحات ضد Brucella melitensis.

الكلمات المفتاحية: مستضدات البروسيلا، الجسيمات النانوية، تحاليل انحلال الدم، مؤشر انحلال الدم.

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