FULL LENGTH | Iranian Biomedical Journal 29 (4): 228-235 July 2025

Apoferritin-Functionalized with Wheat Germ Agglutinin and Loaded with Antibiotic for Targeting Bacteria

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OPEN ACCESS

Article type: Research Article
Received: May 17, 2025
Revised: June 18, 2025
Accepted: June 24, 2025
Published online: June 25, 2025

How to cite:
Gyurjyan QG, Pohosyan GG, Gasparyan VK. Apoferritin-Functionalized with Wheat Germ Agglutinin and Loaded with Antibiotic for Targeting Bacteria. *Iran. Biomed. J.* 2025; 29(4): 228-235.

ABSTRACT

Background: The selective delivery of drugs to their targets prevents their possible side effects; hence, the development of selective transport systems is considered extremely promising. In the present study, we aimed to develop a drug delivery system for targeting bacteria.

Methods: Functionalization of apoferritin with WGA, as a bacteria-recognizing lectin, was conducted. Afterwards, the complex was loaded with ampicillin. The affinity of the conjugate to Gram-positive bacteria, *B. subtilis*, was evaluated by anisotropic AgNPs conjugated with this complex, and its interaction with the bacteria was also assessed.

Results: The drug-delivery capabilities of the composite were evaluated. Results from the disk diffusion assay revealed that its bactericidal activity is 10-fold greater compared to free antibiotics. The effectiveness of the bactericidal effects of ampicillin-loaded ferritin was also confirmed in whole blood.

Conclusion. Lectin-conjugated and ampicillin-loaded apoferritin can be considered as effective drug delivery systems for targeting bacteria.
DOI: 10.61882/ibj.5039

Keywords: Ampicillin, Bacteria, Ferritins, Wheat germ agglutinins

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INTRODUCTION

Infectious diseases remain one of the leading causes of mortality worldwide, and antibiotic resistance represents a major global health challenge. Currently, drug-resistant infections are responsible for at least 700,000 deaths annually, and this figure is projected to rise to 10 million by 2050[1]. Resistance mechanisms often make standard antibiotic dosage ineffective[2], and increasing the dosage does not always lead to improved therapeutic outcomes.

Traditional methods of drug administration have several limitations that can reduce treatment efficacy. In this context, the hydrophobic nature of certain drugs may cause precipitation in the bloodstream, which reduces their bioavailability and solubility. Additionally, many drugs strongly bind to plasma proteins, resulting in the reduced concentration of free active drug available for therapeutic action[3]. In some cases, achieving sufficient efficacy requires high drug concentrations, which leads to serious side effects. Given the time-consuming and costly process of developing new antibiotics, improving the performance of existing drugs through advanced delivery systems or formulation strategies would be a promising alternative. One practical approach to increase the drug efficacy is to develop transport systems that selectively deliver drugs to their targets[4-6]. These carriers have been shown to significantly influence drug distribution, reduce toxicity, and improve therapeutic outcomes. Moreover, they enhance the pharmacokinetic properties of drugs[7-10]. Among the advanced systems, liposomes are well-recognized as effective drug carriers in both pharmaceutical and cosmetic applications[11-14]. Consistently, polymyxin B-loaded liposomes have been successfully employed to treat *P. aeruginosa*-related infections[15]. Treating pneumonia caused by this bacterium presents significant clinical challenges;

List of Abbreviations:
AgNP: silver nanoparticle; **AWA:** apoferritin-WGA-ampicillin conjugate; *B.* subtilis: *Bacillus* subtilis; **CFU:** colony-forming unit; ***P. aeruginosa*:** *Pseudomonas aeruginosa*; **WGA:** wheat germ agglutinin

[DOI: 10.61882/ibj.5039]

however, studies have shown that liposome-encapsulated antibiotics can improve bacterial eradication compared to free drug formulations. Furthermore, an advanced drug delivery system can significantly enhance drug stability and therapeutic efficacy. Research has revealed that ampicillin-loaded liposomes exhibit enhanced stability and higher antimicrobial activity against listeriosis and salmonellosis compared to free ampicillin[16], although liposomes have some drawbacks. In other words, they often face challenges with physical stability, and also, sterilization processes can reduce their integrity, making their use in injectable formulations more complicated. When administered orally, liposomes are susceptible to degradation by digestive enzymes, such as pepsin and bile salts, which can destroy the encapsulated drug before it reaches its target. Moreover, their relatively large size limits their permeability across intestinal epithelial barriers, reducing their effectiveness in targeted drug delivery.

An alternative and promising drug carrier is ferritin, a naturally occurring iron storage protein. By removing its iron core, ferritin is converted to apoferritin, which can be loaded by therapeutic agents without the need for chemical modifications that might alter the structure of the drug. Ferritin can serve as a carrier for drug molecules and has been successfully used to target various tissues. It has demonstrated significant potential in tissue-specific drug delivery, as it is recognized by the transferrin receptor, which is often overexpressed in tumor cells[17]. Clinical studies have displayed that native human ferritin can distinguish cancerous from normal cells with a sensitivity of 98% and specificity of 95% across samples from patients with nine distinct cancer types[18]. Furthermore, human ferritin can effectively target tumors following intravenous injection and is cleared from healthy tissues, indicating favorable pharmacokinetics and biocompatibility with minimal side effects[19,20]. However, in some cases, transport systems may need modification with ligands that recognize alternative targets. Lectins—proteins that bind to carbohydrates—are particularly promising in this regard. Cancer cell membranes often exhibit abnormal glycosylation patterns[21,22], making lectins suitable for both diagnostic and therapeutic applications. In this regard, WGA, a lectin derived from cereal seeds, has been used as a cancer-targeting agent. Studies have demonstrated that paclitaxel-loaded polymeric nanoparticles conjugated with WGA achieve significantly greater therapeutic efficacy than the free drug[23,24]. While most research on targeted drug delivery has focused on cancer therapy, it is important to investigate its potential for treating other diseases.

Selective drug delivery offers a promising approach for treating bacterial and viral infections by allowing the use of potent medications while minimizing side effects. Aguilera-Correa et al. employed mesoporous silica nanoparticles loaded with antibiotics to treat osteomyelitis caused by methicillin-resistant *Staphylococcus aureus*[7]. Similarly, Fereira and colleagues identified liposomes as a promising nanotechnological strategy to combat antimicrobial resistance[25]. Targeted delivery systems have also been explored for viral infection. Galactose-functionalized mesoporous silica nanoparticles have been applied in the treatment of hepatitis C[26]. Current therapies for this infection typically involve a combination of drugs; however, their efficacy is minimal (about 30%), and they are often associated with serious negative effects. Nanoparticle-based transport systems are increasingly being applied for drug delivery, with or without targeted recognition capabilities. For instance, ciprofloxacin-loaded mesoporous silica nanoparticles have been utilized to treat intravascular *Salmonella* infections[27], allowing for a reduced antibiotic dosage compared to the standard treatments. However, these systems primarily rely on passive delivery mechanisms, with more effectiveness resulting from the improved pharmacokinetics and enhanced drug stability.

To improve the precision of the targeted drug delivery, integrating pathogen-recognition elements into transport systems is a promising strategy. Lectins—carbohydrate-binding proteins—are particularly effective due to their ability to recognize specific glycan structures on microbial surfaces. Our research group has previously demonstrated that lectins can effectively detect a broad range of pathogens, including Gram-positive and Gram-negative bacteria, as well as fungi[28-30]. These findings support the use of lectins as targeting ligands in drug delivery platforms, enabling selective and efficient delivery of therapeutics to microbial cells. In this study, we present, for the first time, a novel approach involving ferritin nanoparticles loaded with ampicillin and conjugated with WGA, a lectin known for its strong affinity to bacterial cell walls. We evaluated the binding specificity of this formulation to *B. subtilis*, a Gram-positive bacterium, and compared its antibacterial efficacy to that of free ampicillin.

MATERIALS AND METHODS

Materials

Bovine apoferritin and WGA were supplied by BIO-VAR (Armenia). The *B. subtilis* bacterial strains were provided by Armbiotechnology (Armenia). Ampicillin powder was obtained from Synthesis (Russia), and HW60Toyopearl gel from ToyoSoda (Japan).

[DOI: 10.61882/ibj.5039]

Photometric quantification of ampicillin and synthesis of anisotropic AgNPs

The quantitative determination of ampicillin was performed using a spectrophotometric method based on the formation of a product through acid degradation of the antibiotic[31]. Anisotropic AgNPs were synthesized according to the protocol described earlier[28]. Briefly, nanoparticle seeds were prepared by reducing silver nitrate with sodium borohydride. These seeds were subsequently added to a growth solution containing trisodium citrate, silver nitrate, and ascorbic acid. The appearance of a blue color confirmed the successful formation of anisotropic AgNPs.

Bacterial cultivation and quantification

Bacteria were cultivated on nutrient agar medium (Difco, USA) at 37 °C for 24 hours. The total bacterial concentration was estimated by measuring optical density at 600 nm using a previously described method[32]. This method employs McFarland standards as reference solutions with specific turbidity levels that correlate with defined concentrations of *Escherichia coli* cells per milliliter. Viable bacterial counts were determined using nitro tetrazolium blue, as explained earlier[33]. This assay relies on the metabolic activity of live bacteria, which reduces nitro tetrazolium blue to a dark blue formazan. The intensity of the resulting color acts as an indicator of the presence of viable bacteria.

Ferritin-WGA conjugate for ampicillin loading

Ferritin is a glycoprotein that was functionalized with WGA using a well-known carbohydrate-protein conjugation method[34]. In brief, apoferritin was treated with sodium-m-periodate to generate active aldehyde groups in carbohydrate moieties of ferritin. WGA was then added, and the mixture was incubated in a carbonate buffer (pH 9.5) for 24 hours. This incubation enabled the binding between the aldehyde groups of apoferritin and the free amino-groups of WGA, resulting in the formation of a ferritin-WGA conjugate. Following conjugation, ampicillin was loaded into the conjugate. The pH of the ferritin-WGA solution (4 mg/ml) was lowered to 2.5 through dialysis against a 20 mM glycine-HCl buffer to induce the denaturation of apoferritin. Ampicillin (5 mg), dissolved in the same buffer, was added to the conjugate. After a 30-minute incubation, the pH of the solution was adjusted to 7.4 by adding 0.5 M Tris buffer, which promoted the reassembly of apoferritin and encapsulation of ampicillin. The final mixture was purified using an HW60Toyopearl column equilibrated with PBS (pH 7.3), effectively separating the ferritin-WGA-ampicillin conjugate from the unbound components. Quantitative analysis revealed that the final conjugates contained approximately 10-15 ampicillin molecules per mole of protein. These steps are illustrated in Figure 1.

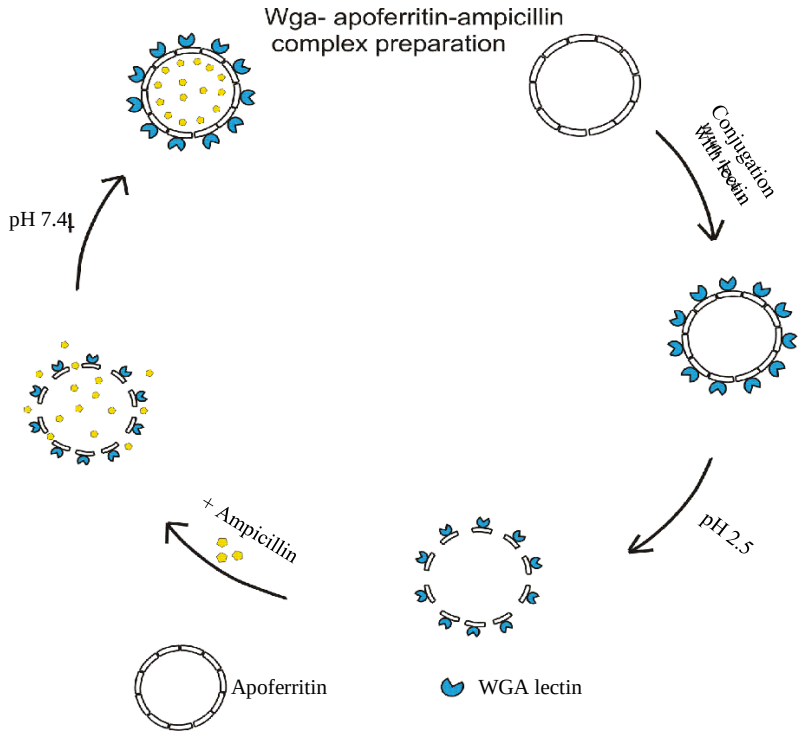

Fig. 1. Schematic representation of apoferritin conjugation with WGA, followed by pH-dependent loading of ampicillin into the resulting conjugate.

[DOI: 10.61882/ibj.5039]

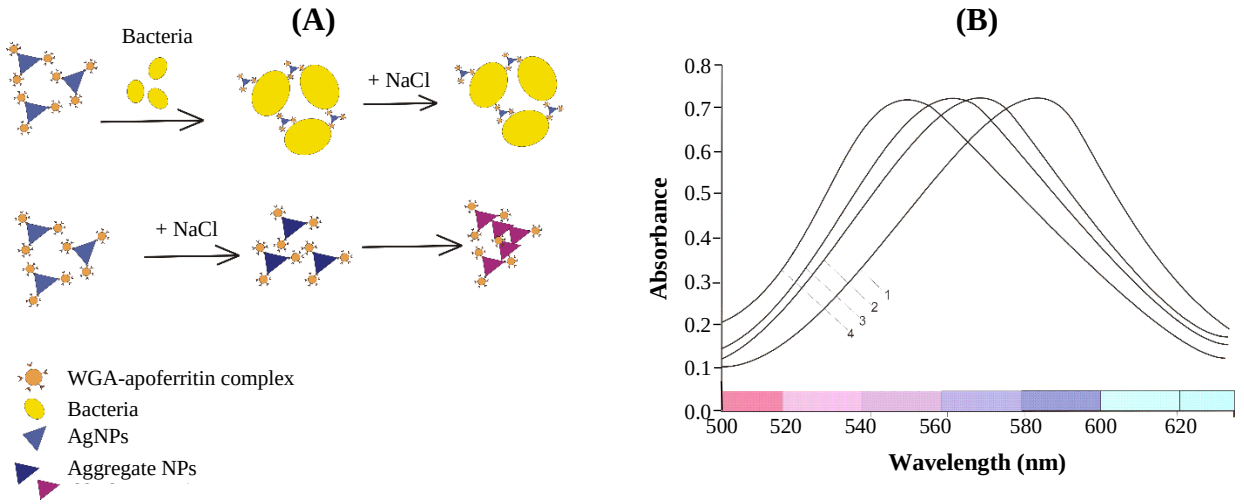

Fig. 2. Detection principle of bacteria binding to AWA-sensitized AgNPs. (A) Schematic representation of the assay using apoferritin-WGA-sensitized AgNPs; (B) Optical changes observed in apoferritin-WGA-sensitized AgNPs in the presence of various concentrations of bacteria: 1: 1×10^6; 2: 2×10^5; 3: 4×10^4; 4: no bacteria.

Sensitization of AWA with anisotropic AgNPs

Borate buffer (0.2 M; pH 8.2) was added to the anisotropic AgNPs to achieve a final concentration of 1 mM. Subsequently, the AWA conjugate was added to the nanoparticle solution at a final concentration of 1 µg/ml. The mixture was then incubated at room temperature for 24 hours. Afterwards, bovine serum albumin (50 mg/ml) was added to achieve a final concentration of 0.15%, and the solution was incubated for an additional 2 hours. Finally, borate buffer was supplemented to adjust the final concentration to 10 mM[28-30].

Statistical analysis

To investigate the reproducibility, the assays were repeated in triplicate. The within-run coefficients of variation were calculated using three measurements, resulting in a coefficient of variation of approximately 9%.

RESULTS

Affinity of the conjugate to the bacteria

To evaluate the binding ability of the conjugated lectin, we assessed its interaction with bacterial cells. This assessment was conducted by examining the affinity of bacteria for AWA using an isotropic AgNPs sensitized with AWA. These anisotropic AgNPs exhibited two distinct plasmonic resonance bands at wavelengths of 400 and 656 nm. Notably, the longer wavelength band was sensitive to surface changes, which can include alterations in the dielectric interparticle distance and nanoparticle aggregation[28]. Therefore, any interaction between AWA-sensitized nanoparticles and bacteria is expected to induce significant changes in the optical properties of the nanoparticles. As illustrated in Figure 2A, the assay begins with the interaction between bacteria and AWA-sensitized AgNPs. After this interaction, a 2 M NaCl solution was added to achieve a final concentration of 0.1 M. When bacteria were present, nanoparticle aggregation was inhibited. In the absence of bacteria or at low bacterial concentrations, aggregation occurs. This aggregation led to a shift in the optical spectra, especially a transition from the long-wavelength plasmonic band toward shorter wavelengths. These spectral changes were directly correlated with bacterial concentration. Figure 2B illustrates the effect of varying bacterial concentrations on the optical properties of AWA-sensitized AgNPs. The findings demonstrate that AWA complexes effectively recognize bacterial cells, indicating their potential for targeted antibacterial therapy.

Bactericidal activity of the conjugate

In our study, 1 ml of sterile human blood from healthy donors was inoculated with *B. subtilis* at a final concentration of 10^7 cells/ml and incubated at 37 °C for 24 hours. The absorbance at 600 nm and CFUs varied slightly across bacterial species, and an OD_{600} of 1 corresponded to less than 10^7 CFU/ml[33]. This detection sensitivity is clinically relevant, as UTIs are commonly diagnosed when bacterial loads exceed 10^5 CFU/ml[35]. Subsequently, free ampicillin and ampicillin-loaded ferritin-WGA complexes were added to the blood samples. After an additional 24-hour incubation, the samples were centrifuged at 3000 ×g for 20 minutes to

[DOI: 10.61882/ibj.5039]

Table 1. Comparative antibacterial activity of free and apoferritin-WGA-loaded ampicillin based on *B. subtilis* colony formation

Ampicillin (µg)	Number of colonies	Amount of ampicillin in AWA complex (µg)	Number of colonies
1	420	1	50
2	136	2	0
3	5	3	0

remove erythrocytes. The resulting plasma was then cultured on nutrient agar, and bacterial colonies were assessed after 24 hours. The data demonstrated that 2 µg of ampicillin, when delivered via the ferritin transport system, completely inhibited the colony-forming ability of bacteria. In contrast, the same amount of free ampicillin had no effect (Table 1). Figure 3 shows a higher number of dead bacteria when using the conjugate compared to the free antibiotic.

DISCUSSION

Selective drug delivery for infectious diseases is a promising strategy in pharmacology, as it minimizes side effects, reduces therapeutic doses, and preserves the native structure of the drug after administration. Such an approach in the treatment of infectious diseases is increasing due to the elevated cases of bacterial resistance to drugs[36].

Recent research in antibiotic delivery systems has focused on developing carriers that protect drugs and enhance their pharmacokinetic profile.

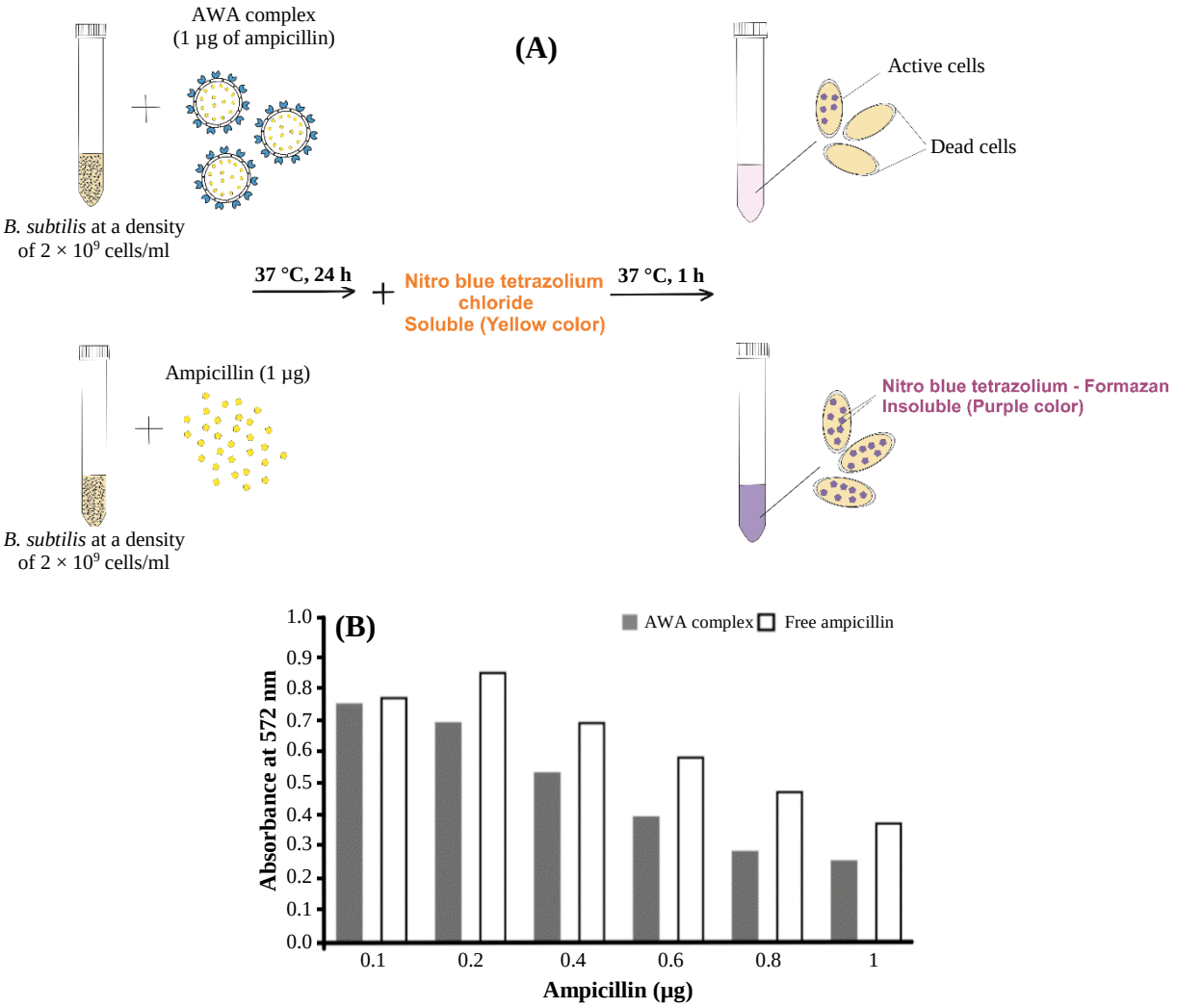

Fig. 3. (A) Quantification of *B. subtilis* cells via nitrotetrazolium blue analysis; (B) the difference in antibacterial effectiveness between conjugated antibiotic and its free form. Increasing the antibiotic concentration results in a more considerable disparity in their effects.

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Abo-Neima and colleagues have demonstrated that functionalized multi-walled carbon nanotubes exhibit strong antimicrobial activity against both *E. coli* and *S. aureus*[37]. Similarly, Jiang and colleagues developed a polyethylene imine-modified graphene oxide platform for tetracycline delivery[38], achieving a fourfold increase in antibacterial efficacy against *S. aureus* and *E. coli* compared to free antibiotics.

Some delivery systems incorporate bacterial recognition ligands to improve targeting. In this context, Le et al. showed that antibody-functionalized nanoparticles could significantly enhance in vitro bactericidal activity against *S. aureus* in both planktonic and biofilm forms[39]. In a mouse model of biofilm infection, these antibody-loaded nanoparticles demonstrated superior treatment efficacy to free antibiotics. However, the use of antibodies is costly and limited to specific bacterial strains. Antibodies are highly specific ligands that bind with high affinity to the antigens present on the bacterial cell surfaces. However, such ligands are very expensive, and they are only specific to certain bacteria. Lectins present a more universal alternative, as they can recognize both Gram-positive and Gram-negative bacteria, making them suitable ligands for targeted delivery. Hence, they can be used as bacteria recognizing ligands in all transport systems. Moreover, ferritin-based drug delivery systems offer several advantages over liposomes and synthetic drug delivery systems. Ferritin-based carriers are non-toxic, do not form protein coronas, and are less prone to clearance by the reticuloendothelial system. Therefore, sensitizing ferritin with lectins enhances specificity, while allowing efficient drug loading without covalent modifications that could compromise drug activity. In comparative studies, the bactericidal activities of free antibiotics and ferritin-loaded antibiotic complexes were evaluated against *B. subtilis* in blood samples. The results demonstrated that the ferritin-based system significantly exhibited greater antibacterial efficacy in both blood and culture conditions, highlighting its potential for targeted antimicrobial therapy. Our data demonstrated the high efficiency of the WGA-modified ferritin loaded with an antibiotic for selective drug delivery towards bacterial cells, and are promising for wider application in other systems.

CONCLUSION

A conjugate of WGA with apoferritin was synthesized and loaded with ampicillin. This apoferritin-WGA conjugate demonstrated a selective binding to *B. subtilis*, a Gram-positive bacterium. When tested in blood samples and bacterial cultures, the conjugate exhibited significantly enhanced bactericidal activity compared to free ampicillin. Notably, the efficacy of this targeted delivery system was approximately 10-fold greater than that of the free antibiotic. Lectins, such as WGA, can serve as promising, cost-effective ligands for bacterial recognition. Their broad applicability across different transport platforms makes them attractive candidates for developing advanced antimicrobial delivery systems.

DECLARATIONS

Acknowledgments

No artificial intelligence services were used for the preparation of this manuscript.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

QGG: investigation and methodology; GGP: investigation; VKG: methodology, conceptualization, and writing the manuscript;

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by a scientific grant of the State Committee on Science of the Ministry of Education and Science of the Republic of Armenia [grant no. 21SC-RBSCST-1F001].

Supplementary information

The online version does not contain supplementary material.

[DOI: 10.61882/ibj.5039]

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[DOI: 10.61882/ibj.5039]