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A Review of Methodological Standards and Current Practices in Hemolytic Toxicity Testing of Nanoparticle-Based Drug Delivery Systems

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Hemolytic toxicity testing is a fundamental component of hemocompatibility assessment for nanoparticle-based drug delivery systems (N-DDSs), as erythrocyte integrity reflects blood safety prior to clinical application. Despite its critical role, experimental procedures vary across the literature. Essential methodological details are frequently omitted, hindering reproducibility and standardization. This variability urges the need for a consolidated reference that examines both current practices and established guidelines. This narrative review aims to provide a structured overview of hemolytic toxicity testing methodologies applied to N-DDSs, with particular emphasis on procedural parameters and their alignment with ASTM and ISO standards. Literature was identified through Google search and open access Scopus-Indexed journals primarily covering publications from 2025 and supplemented with earlier studies to provide historical context. Articles were selected based on relevance to seven methodological parameters which were erythrocyte source, methods of erythrocyte separation and purification, incubation conditions, positive and negative controls, measurement method. Analysis of 30 publications revealed human erythrocytes were the predominant model, with phosphate buffered saline and detergent-like substances were most commonly used negative and positive controls, respectively. Centrifugation controls were inconsistently reported, while incubation was typically run at 37°C, with varying durations depending on the nanoparticle system. Variability was also observed in approaches used to quantify free hemoglobin. Overall, while hemolytic toxicity studies continue to rely on ASTM and ISO guidelines, greater methodological details and standardization would enhance the reliability and reproducibility of hemocompatibility assessments in nanoparticle drug delivery research.

INTRODUCTION

Biocompatible means the harmonious nature of life entities existing together (Crawford et al., 2021), whilst its measure is the 'biocompatibility'. The latter is a crucial aspect in the development of drugs, dosage forms, and drug delivery systems (DDS), particularly when its intended applications involve contact with red blood cells (RBC) or erythrocytes. Erythrocytes are a major component of our circulation (Sherwood, 2016) and have been a subject for toxicity evaluations of nanoparticle-based drug delivery systems (N-DDSs).

Drug delivery systems designed to be distributed through our circulation system are destined to interact with erythrocytes. Moreover, since erythrocytes are the main composition of whole blood, they are incoherent to eliminate, in the favor of administrating drug delivery systems. Rather, a solution would be to design the DDS by means of maintaining

erythrocyte integrity. This strategy has been implemented in previous drug delivery system studies (Ansary et al., 2025). The engineered delivery systems are then evaluated for their safety through possible hemolytic activity, since interaction of nanoparticles with erythrocytes can lead to morphological changes of these cells, causing hemolysis (Azevedo et al., 2024; Mazzarino et al., 2015). This approach to study biocompatibility of nanoparticles was highlighted in the early 2000s. (Dobrovolskaia et al., 2008)

Hemolysis itself is the release of hemoglobin from erythrocytes following its rupture. Certain disease conditions (Dubach et al., 2025), infections (Deng et al., 2022), or drugs and toxins (Zheng et al., 2025) may lead to hemolysis. Besides pathophysiological causes, the size, shape, and surface charge of nanoparticles play a role in determining the destiny of erythrocytes. Results are influenced by factors including nanoparticle size, coating, charge, and experimental conditions; as studied in the references used in **Table 1**.

The lysis of erythrocytes within the human body in turn, gives rise to numerous clinicopathological disorders (Dvanajscak et al., 2019) which propose risks to the patient. Therefore, we evaluate the hemolytic activity potential of materials through hemolytic toxicity testing (HTT), to conclude their hemocompatibility.

A N-DDS can be considered biocompatible when no adverse effects resulting from its interaction is declared. Although HTT is critical for evaluating the biocompatibility of nanoparticle-based drug delivery systems, experimental procedures and methodological specifications are often incompletely reported in the literature. This lacking, complicates reproducibility and limits meaningful comparison across studies, despite the availability of ASTM and ISO guidelines. Commonly omitted details include, erythrocyte source, erythrocyte separation, erythrocyte purification, incubation condition, control selection, and basis for measurement method. As a result, researchers are frequently required to conduct extensive literature searches to construct experimental protocols. Therefore, this review aims to provide a comprehensive and methodologically focused reference of recent hemolytic toxicity studies, comparing current practices and their alignment with existing guidelines to support a more consistent and producible biocompatibility assessment of nanoparticle-based drug delivery systems.

METHODS

The present article focuses on the methodological aspects of hemolytic toxicity studies of nanoparticles as drug delivery systems, conducted as a narrative review. The literature screening and selection process is shown in **Figure 1**.

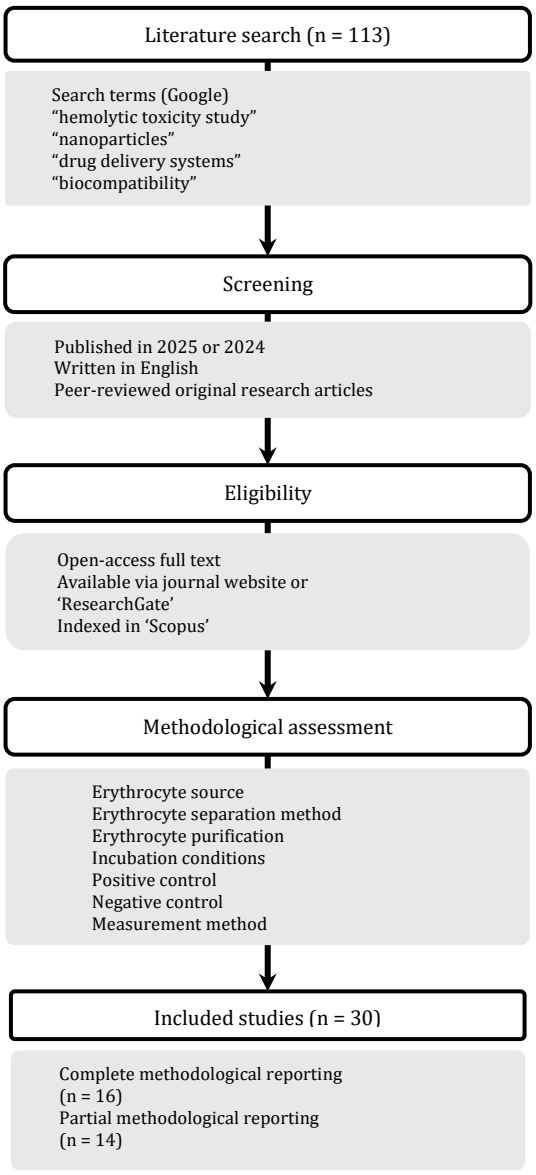

Figure 1. Literature screening and selection process for the narrative methodological review

Relevant publications were sourced through Google search, mainly covering year 2025. Articles were filtered using combinations of the search terms "*hemolytic toxicity study*", "*nanoparticles*", "*drug delivery systems*", and "*biocompatibility*". Priority was given to peer-reviewed original research articles, written in English. Only full-text open access articles available on journal websites, or uploaded by the authors through ReasearchGate, and indexed in Scopus were considered. Earlier studies and recent guidelines were incorporated to provide historical context. The final selection of articles was based on their relevance to seven key methodological parameters, which are erythrocyte source, methods of erythrocyte separation and purification, incubation conditions, positive and negative controls, and measurement method. Among the 30 publications included in this review, 16 provided complete information on all parameters. Studies with incomplete methodological details were nevertheless retained, as the information they contained contributed valuable insights into current practices and preferences in hemolytic toxicity testing. Concordance of reported methods with standard, current guidelines are also discussed. This approach consolidates methodological knowledge and practical considerations into a single comprehensive reference for researchers conducting hemolytic toxicity studies on erythrocytes.

RESULT AND DISCUSSION

Hemolytic toxicity study principle

A widely used method to evaluate the biocompatibility of substances is through hemolytic toxicity. Materials predicted to have hemolytic activity is incubated with pre-washed red blood cells (erythrocytes). If the material is hemolytic, erythrocytes undergo hemolysis, releasing the iron conjugated protein, hemoglobin. Hemoglobin is separated, and its absorbance is directly measured using a spectrophotometer. The direct method proceeds to measuring the hemoglobin straight away. Whilst the addition of Drabkin's reagent which converts hemoglobin to cyanhemoglobin before measurement, is distinguished as the indirect method. The degree of hemolysis of the substance is then compared to positive and negative controls.

It is important to not be confused by the various terms referring to hemolytic toxicity study, including red blood cell cytotoxicity, hematoxicity, hemocompatibility testing, test of hemolysis (Rojas-Mancilla et al., 2015), and hemolysis test (Ansary et al., 2025), In one study, besides hemolysis toxicity assessment, hemocompatibility also covered whole blood clotting study (Aroonthongsawat et al., 2025). In this review the term 'Hemolytic Toxicity' will be used.

Categories of hemolysis

The American Society for Testing and Materials (ASTM), issued in 1993, was one of the first Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM F 756-00) available. (ASTM International, 2000). Almost over the last decade, researchers in the fields of nanomaterial and nanoparticle hemocompatibility (Shakibaie et al., 2025; Sharifi et al., 2025) have refered to its revision, ASTM F756-08, issued in 2008 (ASTM International, 2008, and most currently, ASTM F756-13, issued in 2013 as the 'Standard Practice for Assessment of Hemolytic Properties of Materials' (ASTM International, 2013) .The ASTM F756-13 guidelines are in line with ISO/TR 7406, a technical report ellaborating the biological evaluation of medical devices, as a guidance for hemocompatibility testing related to blood-material interactions (International Organization for Standardization (ISO), n.d.)., which complements ISO 10993-4. The Food and Drug Administrative (FDA) has also endorsed, biocompatibility guidelines by ASTM and ISO (US FDA, 2023). Either guidelines have been referred to by many researches on determining hemocompatibility (Ansary et al., 2025; Salimi et al., 2025; Shakibaie et al., 2025; Sharifi et al., 2025).

Hemolysis classification of substances are mentioned in the guidelines. Non-hemolytic activity is presented by hemolysis percentage of below 2%, and slightly hemolytic if 2 – 5%. While 5% hemolysis findings indicate hemolytic activity, suggesting potential harm to red blood cells. In other words, a hemolysis percentage below 5% implies hemocompatibility of the substance (Peng et al., 2025). The value is

considered the critical safe hemolytic ratio for biomaterials, and hence, non-hemolytic (Aroonthongsawat et al., 2025; Mazzarino et al., 2015)

Erythrocytes as model cells

The human circulation system is accounted by whole blood which consists more than half of erythrocytes who carry a crucial role as a vehicle for oxygen transport (Sherwood, 2016). Erythrocytes have thin cell walls which facilitates exchange of oxygen. Their membranes are flexible to mediate their access and flow through extremely narrow blood capillary measuring below 3 μm (Canham & Burton, 1968). Although erythrocytes are highly deformable due to their flexibility, they are fragile and prone to hemolysis. Excessive stretching, which increases membrane surface area, can compromise membrane integrity and and promote cell rupture. Deformability is therefore a key in erythrocyte quality and can be adversely affected by factors such as prolonged storage duration and exposure to shear stress (Canham & Burton, 1968; Ebrahimi & Bagchi, 2022; Islamzada et al., 2022; Jeong et al., 2006)

Additionally, erythrocytes are ideal models for cytotoxicity evaluation in biocompatibility studies of nanoparticles. Besides being accessible, literature regarding their characteristics and stability is abundant. Erythrocytes are sensitive to environmental physical and osmotic alterations which can result in their lysis. Blood poisoning as a manifestation of released hemoglobin into the circulation post administration of nanoparticles, due to hemolysis is a signal of some extent of toxicity (Mazzarino et al., 2015; Yedgar et al., 2022).

Various specimens in addition to human erythrocytes have been used for hemolysis evaluation, including rabbit (Huang et al., 2025), rat (Peng et al., 2025; Qian et al., 2025), and mice (Ilhami et al., 2025; Shi et al., 2025)whole blood. However, human erythrocytes are the most widely used as they provide the most clinically relevant model for predicting hemolytic outcome in humans. Therefore, when specimens from alternative species are employed, it is important to account for differences in blood composition, particularly erythrocyte composition, as well as its stability and integrity with respect to pH and osmotic environment conditions.

Ex-vivo procedures isolate erythrocytes in the form of fresh whole blood, whilst in-vitro procedures generally involve application of cell lines or artificial erythrocyte models. The standard method used is the ex-vivo method since it can present the actual conditions of erythrocytes. Nonetheless the term in-vitro hemolytic toxicity study is frequently used even though the cells originate from donors or laboratory animals.

Hemoglobin

Enclosed inside erythrocytes, attached to iron (Fe), hemoglobin, are globular proteins comprising four subunits. These hemoglobins are the actual analyte to be measured by the instrument, and their release indicates hemolytic potential of the certain nanoparticles. Hemoglobin can be detected by spectrophotometric methods due to the many conjugated and double bonds, primarily owned by the heme group of hemoglobin

Absorbance of hemoglobin at has been reported to have maximum absorbance at a wavelength ranging from 370 – 655 nm. This difference can be due to slight alterations in solvent pH, use of cyanohemoglobin reaction, or oxidation state of hemoglobin. Common hemoglobin derivatives include, but may differ from literature on the abbreviation are, oxyhemoglobin (Hb O_2), carboxyhemoglobin (HbCO), methemoglobin (Hi), and sulfhemoglobin (SHb). Oxyhemoglobin (HbO_2) is bound to oxygen, deoxyhemoglobin (Hb) is not oxygen bound, and methemoglobin (Hi) is an oxidized form. Various spectrophotometric methods have been used which involves two-wavelength methods. Such methods are relatively tedious which may need pretreatment to produce two species which can absorb radiation at different wavelengths. Whilst, for determining the total hemoglobin, conversion of hemoglobin to cyanhemoglobin (HiCN) is usually done artificially through the reaction with Drabkin's reagent (containing $K_3Fe(CN_6)$). This forms a stable hemoglobin derivate which can be directly measured by the spectrophotometer at 540 nm (van Kampen & Zijlstrat, 1983), and is a

more practical approach. A summary of wavelengths and instruments used in recent studies can be seen in **Table 1**.

Hemolytic Toxicity Study Procedure

One of the earliest quantitative assessments of nanoparticle-induced hemolytic toxicity was reported by, Dobrovolskaia et al., (2008) employing the ASTM F756-00 standard protocol. Prior to this, most investigations were qualitative in nature and lacked adherence to standardized methodologies, limiting comparability across studies. In 2018, Neun et al., 2018, further advanced methodological rigor by underscoring the necessity of validating whole blood quality prior to hemolysis testing. This recommendation is critical, as the presence of pre-hemolyzed erythrocytes can artificially elevate measured hemolysis values, thereby generating false-positive results and compromising the reliability of the assay. This development made performing validation of whole blood quality essential prior to the study.

Whole blood collection

Whole blood is obtained in an anti-clot blood collecting tube which prevents the specimen from clotting. Appropriate sampling containers can comprise of lithium-heparin blood tubes (Shakibaie et al., 2025), K2-EDTA blood tubes (Chan et al., 2025; De Menezes et al., 2014; Sohail et al., 2025; Zhaisanbayeva et al., 2024), and EDTA tubes (Ben Amor et al., 2025). In cases where not used immediately, whole blood can be stabilized through addition of citrate phosphate double dextrose Solution (CP2D) as an anticoagulant (Bernier et al., 2021), and whether stabilized or not, stored at lower temperatures, commonly at 4^{o}C. Nonetheless it is recommended to immediately run hemolytic toxicity studies after whole blood collection and erythrocyte separation (Kuck et al., 2025; Uyuklu et al., 2009)

Erythrocyte isolation

Acceleration of plasma separation from erythrocytes is typically achieved by centrifugation. The plasma is used for specimen validation while the erythrocytes are used for the study. In the literature (**Table 1**), centrifugation speed is majority reported in revolutions per minute (rpm) rather than relative centrifugal force (g), which can hinder reproducibility across studies. Optimization of centrifugation parameters, is essential to minimize the risk of procedure-induced hemolysis and to ensure the integrity of the erythrocyte at separation stage in specimen validation and after incubation.

Validation of whole blood quality

Subsequently, after centrifugation, the supernatant (plasma) is quantified for free hemoglobin content. The concentration of hemoglobin present, is determined spectrophotometrically. A lower measured concentration indicates low levels of free hemoglobin in the plasma, reflecting minimal hemolysis and greater erythrocyte integrity. For application in hemolytic toxicity studies, whole blood is considered acceptable only when plasma free hemoglobin concentration is no more than 1 mg/mL (Neun et al., 2018). This value should be taken account for during erythrocyte purification, which the latter is generally done 2 – 3 times (Aroonthongsawat et al., 2025; Chan et al., 2025), or until the supernatant is free from absorbance (Ilhami et al., 2025; Salimi et al., 2025; Shi et al., 2025). The quantification of free hemoglobin is possible through applying a standard calibration curve (Neun et al., 2018).

Erythrocyte dilution

After plasma removal, the erythrocytes are purified with phosphate buffered saline (PBS). Adjusting to the spectrophotometer's measurable concentration range, erythrocytes are diluted before incubation with the sample. Dilution level is determined so that the hemoglobin's absorbance can fall within the spectrophotometer's measurable range, where the standard dilution is about 200 – 250 times (Huang et al., 2025; van Kampen & Zijlstrat, 1983). To ensure erythrocyte integrity and purity before exposure to the hemolytic agent, dilution and washing procedures should be performed in a medium that simulates the composition of whole blood, in terms of tonicity, and pH. This is achieved with reagents namely, phosphate buffered saline (PBS) with a pH 7.4 (Ansary et al., 2025; Sohail et al., 2025), PBS with a pH 7.3 (Ullah et al., 2025), and saline (Chan et

al., 2025), with most frequently chosen is PBS pH 7.4.

Sample incubation

A suspension of diluted erythrocyte is incubated with the nanoparticle sample at various concentrations, meaning only two components are within the incubation tube. Sample preparation is commonly done in triplicate (Peng et al., 2025). Incubation temperature is set to mimic human body temperature (37°C), while incubation periods vary depending on the analyte, analyte target, and prediction of circulation or erythrocyte contact duration inside the human body. Period of incubation may range from minutes to hours. Whether short or long periods, gentle shaking at designated time intervals or through-out the incubation period will aid a homogenous suspension to optimize interaction time between erythrocyte and analyte. (Aroonthongsawat et al., 2025; de La Taille et al., 2025; Mazzarino et al., 2015; Sharifi et al., 2025). Several studies also supplement incubation with a concentration of 5% CO_2 (Bernier et al., 2021; Ilhami et al., 2025) to create real physiological conditions.

Positive and negative controls

Positive controls for hemolytic toxicity studies must be able to induce hemolysis. According to the ASTM standards, a substance which can cause hemolysis above 8% is suitable as the positive control (**Figure 2**).

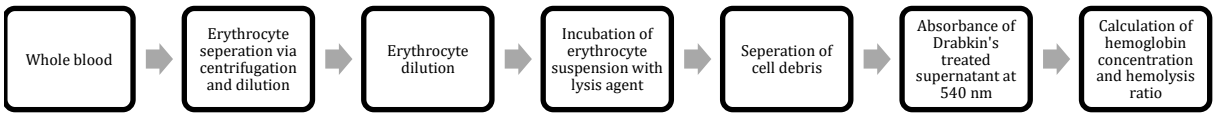

Figure 2. Workflow for hemolytic toxicity testing of samples

These substances, also termed as a 'lytic agent' basically have detergent or surfactant-like properties that effectively disrupt erythrocyte membranes. A widely used positive control is Triton-X 100 at a concentration of 1%, though concentrations of 0.4%, 0.5%, and 20% have been reported (**Table 1**). Alternatives include deionized water, distilled water, sterile water, and ascorbic acid. A solution of 1% Triton-X 100 showed 100% hemolytic activity within 2 hours of incubation, whilst ultrapure water needed 24 hours to induce hemolytic activity (Bernier et al., 2021).

The negative control is expected to produce 0% hemolysis, as it should be isotonic and maintain a physiological pH similar to blood. Phosphate-buffered saline (pH 7.4) is most commonly used, although normal saline is also employed. Although frequently used as the negative control, saline was found to not be completely clear of hemolysis (Qian et al., 2025). In some reports, other solutions such as 0.5% dimethyl sulfoxide (DMSO), Alsever's solution, or even distilled water have been used. These various options must have lower hemolytic activity than the positive control chosen in the study.

Hemolysis observation

Hemolysis can be analyzed qualitively through morphological observation, and quantitatively through absorbance of free hemoglobin. Hemoglobin absorbance measurement using either a spectrophotometer and cuvettes, or a microplate reader is based on the Lambert and Beer Law, hence, samples are required to be transparent and clear (Mayerhöfer et al., 2020).

Erythrocytes observation can be documented after incubation (Na et al., 2025), while the hemolysis ratio is reported in percentage using the formulas below (Aroonthongsawat et al., 2025; Chaurasiya et al., 2025). Formula (1) and (2) yields % of hemolysis, where no calibration curve is required, in contrast to the validation step of the hemolysis assay where a calibration curve is necessary. As (absorbance of sample), An (absorbance of negative control), Ap (absorbance of positive control), ODs (optical density of sample, ODn (optical density of

negative control), ODp (optical density of positive control).

$$Hemolysis\ ratio\ (\%) = \frac{As-An}{Ap-An} \times 100\% \ldots.(1)$$

or

$$Hemolysis\ ratio\ (\%) = \frac{ODs-ODn}{ODp-ODn} \times 100\% \ldots(2)$$

Hemolytic toxicity studies carry procedural variability, which tend to evolve around key methodological parameters, which are, erythrocyte source, methods of erythrocyte separation and purification, incubation conditions, positive and negative controls, and measurement method. The purpose of this review has been to provide a structured narrative overview on hemolytic toxicity studies, with a particular focus on their application in nanoparticle drug delivery system development. The current ASTM and ISO guidelines remain highly relevant, in the aspects of methodology and for hemolysis categorization. Human erythrocytes are the predominant model, as they offer greater clinical relevance than animal-derived cells. However, reproducibility of erythrocyte separation (and removal of cell debris after incubation) is limited by centrifugation conditions, having being commonly reported in rotations per minute (rpm), rather than relative centrifugal force (g). For this reason, optimization of centrifugation protocols is recommended depending on the cell model. As for instance, 3000 rpm may yield inconsistent forces across instruments. Once separated, erythrocytes are generally purified by implementing PBS as the washing solution, which also serves as the most frequent negative control. In contrast, Triton X-100, is widely used as the positive control, providing a standardized value of 100% hemolysis. Incubation conditions typically mimic physiological temperature (37°C), although exposure times vary according to the drug delivery system under study. Finally, a high degree of variability was also observed for the choice of free hemoglobin detection wavelength, nonetheless measurement at 540 nm was the most commonly adopted approach. Alternative wavelengths were determined by the overall methodology, which ultimately influences the final analyte for free hemoglobin calculation. Therefore, standardization of wavelength selection aligned with the methodology and the specific hemoglobin derivative measured, will improve reproducibility and reliability in future hemolytic toxicity studies.

Table 1. Comparison of Methodological Parameters Reported in Hemolytic Toxicity Testing of Nanoparticle-Based Drug Delivery Systems

No.	Erythrocyte source (I)	Erythrocyte separation (II)	Erythrocyte purification (III)	Incubation condition (IV)	+ control (V)	+ control (VI)	ʎ (nm)/ Instrument (VII)	Reference
1	Rat	Centrifugation, resuspended in normal saline	*	30”	*	Saline	541/ Spectrophotometer	(Peng et al., 2025)
2	*	Centrifugation, resuspended in PBS	*	*	Deionized water	PBS	370/ Spectrophotometer	(Shakeel et al., 2024)
4	Rabbit	Centrifugation (3000 rpm, 15’), resuspended in PBS (pH = 7.4)	Washed with PBS (pH 7,4), thrice	37 °C, 4 h	Deionized water	PBS (pH 7,4)	540/ Spectrophotometer	(Ansary et al., 2025)
5	Human	Centrifugation (1000 rpm, 10’), and diluted to a 2% RBC solution	*	37 °C, 1 h	Water	PBS	541and 655	(Zhaisanbayeva et al., 2024)
6	Human	Centrifugation, resuspended in PBS (pH 7.4)	*	37 °C, 1 h	Distilled water	Saline	540/ spectrophotometer	(Medeiros et al., 2025)

No.	Erythrocyte source (I)	Erythrocyte separation (II)	Erythrocyte purification (III)	Incubation condition (IV)	+ control (V)	+ control (VI)	ʎ (nm)/ Instrument (VII)	Reference
7	Human	Centrifugation (3000 rpm, 15')	Washed with PBS thrice	37 ◦ C, 1h, 6 h, invertion every 30'	Distilled water	PBS	540/ spectrophotometer	(Aroonthongsawat et al., 2025)
8	Rat	Centrifugation (1500 rpm, 10')	Washed with PBS (pH of 7.4), thrice	-	Triton X-100	PBS	540/ Microplate reader	(Sohail et al., 2025)
9	Mice	Centrifugation (1500 rpm, 10')	Washed with PBS until supernatant becomes clear	37°C, 4 h	Distilled water	Saline	576/ Microplate reader	(Shi et al., 2025)
10	Rabbit	Centrifugation (3000 rpm, 5')	Washed with PBS, and diluted to a 2% RBC solution	37°C	1% (v/ v) Triton X-100	PBS	540/ *	(Huang et al., 2025)
11	Human	*	*	37 ◦ C, 2 h 300 rpm	1% (v/ v) Triton X-100	PBS (pH 7.4)	420/ *	(Sharifi et al., 2025)
12	Human	Centrifugation (3000 rpm, 10')	Washed until supernatant is transparent	37 ◦ C, 2 h	Triton X-100 (1 %)	PBS	570/ *	(Salimi et al., 2025)
13	Mouse	Centrifugation (12000 rpm, 15')	Washed with PBS 3x until clear supernatant	37 ◦ C, 4 h, 5% CO_2 incubator	Triton X-100 (1 %)	PBS	540/ Microplate reader	(Ilhami et al 2025)
14	-	Centrifugation (1500x g, 10', 4°C)	Washed with NaCl once	37 ◦ C, 1 h	Triton X-100 (20 %)	PBS	450/ *	(Chan et al., 2025)
15	Human	Centrifugation (1000 rpm, 15')	Washed with PBS pH 7.3, thrice	30 °C, 60'	Triton X-100 (0.5%)	DMSO 0.5%	520/ *	(Ullah et al., 2025)
16	Rat	*	*	37 °C, 4 h	Sterile water	Normal saline	545/ *	(Qian et al., 2025)
17	Human	Centrifugation (3000 rpm, 5', room temperature)	RBC washed thrice with Alsever's solution	37 °C, 30'	Deionized water	Alsever's solution	415/ *	(Shakibaie et al., 2025)
18	-	Centrifugation (2000 rpm, 10')	*	37 °C, 3 h	Ascorbic acid	Distilled water	540/ Microplate reader	(Ben Amor et al., 2025)
19	Human	*	*	37 °C, 3 h **	Triton-X100 (0.4%)	PBS	540/ Spectrophotometer	(Kasprzyk et al., 2025)
20	Human	*	*	37°C in a 5% CO_2, 1, 2, and 24 h	1% Triton	PBS	540/ Spectrophotometer	(Bernier et al., 2021)
21	Human	Centrifugation (500xg, 5')	Washed with saline twice, PBS once	37°C, 60', slow agitation	20 % (v/v) Triton X-100	PBS	541/ Microplate reader	(de La Taille et al., 2025)
22	Human	Centrifugation (4000 rpm, 10')	Wached with saline, thrice, and diluted to a	37°C, 1 h, in the dark	Mili-Q water	Normal saline	540/ Microplate reader	(Chaurasiya et al., 2025)

No.	Erythrocyte source (I)	Erythrocyte separation (II)	Erythrocyte purification (III)	Incubation condition (IV)	+ control (V)	+ control (VI)	ʎ (nm)/ Instrument (VII)	Reference
			2% RBC suspension					
23	Human	Centrifugation (3000 rpm, 15')	Washed with PBS	37°C, 4 h, with shaking	Deionized water	PBS	540/ Spectrophotometer	(Shafique et al., 2025)
24	Human	Centrifugation	Washed with sterile PBS thrice,	37°C, 4 h	Deionized water	PBS	540/ Spectrophotometer	(Yaghoubi et al., 2025)
25	*	*	with PBS pH 7,4, twice, and diluted to a 2% RBC suspension	37°C, 24 h	*	*	540/ Microplate reader	(Christodoulou et al., 2025)
26	Human	Centrifugation (1500 rpm)	Washed with 0.9% saline, resuspended to 10% v/v	37°C, 60'	Triton X-100 (1%)	Normal saline	540/ Microplate reader	(Chahardoli et al., 2025)
27	Sheep	*	*	37°C, 30'	Deionized water	PBS	562/ Spectrophotometer	(Ngcamu et al., 2025)
28	*	*	*	37°C, 60'	Double distilled water (ddH_2O)	Normal saline	545/ Spectrophotometer	(Ma et al., 2025)
29	Rat	Centrifugation (3000 rpm, 20')	Washing * Resuspended in normal saline	*	Distilled water	*	541.5*, 576.5*. 545/ spectrophotometer	(Luan et al., 2025)
30	Rat	Centrifugation (4000 rpm, 15')	Washed with PBS, thrice, resuspended to a 2% erythrocyte suspension	37°C, 4 h	1% sodium dodecyl sulfate (SDS) in PBS	PBS	540/ Microplate reader	(Na et al., 2025)

*) Not mentioned/ not detailed in the literature

CONCLUSIONS

Hemolytic toxicity studies remain crucial in the biocompatibility evaluation of nanoparticle-based drug delivery systems, prior to clinical applications. This review highlights ASTM F756 and ISO as relevant guidelines referred to in recent studies, and that methodological variability persists across literature. Standardization of evident variations, mainly for erythrocyte separation and detection wavelength should be prioritized. Moreover, methodology transparency and harmonization, will definitelylead to more effective, reproducible, and comparable hemolytic toxicity studies to support the development of nanoparticle-based drug delivery system.

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AUTHORS' CONTRIBUTIONS

All authors contributed to the research

CONFLICT OF INTERESTS

None.

BIBLIOGRAPHY

Ansary, A., Osman, A., & El-Khouly, M. E. (2025). Doxorubicin-loaded pH-responsive porphyrin-derived carbon dots as a promising biocompatible drug delivery system for effective chemotherapy of breast cancer. *RSC Advances*, *15*(9), 6457–6473. https://doi.org/10.1039/d4ra09058f

Aroonthongsawat, P., Manocheewa, S., Srisawat, C., Punnakitikashem, P., & Suwanwong, Y. (2025). Enhancement of the in vitro anti-leukemic effect of the histone deacetylase inhibitor romidepsin using Poly-(D, L-lactide-co-glycolide) nanoparticles as a drug carrier. *European Journal of Pharmaceutical Sciences*, *207*. https://doi.org/10.1016/j.ejps.2025.107043

ASTM International. (2013). *ASTM F756-13: Standard Practice for Assessment of Hemolytic Properties of Materials*.

ASTM International. (2000). *ASTM F756-00; Standard Practice for Assessment of Hemolytic Properties of Materials*.

ASTM International. (2008). *ASTM F756-08: Standard Practice for Assessment of Hemolytic Properties of Materials Designation* .

Azevedo, N. C. L., de Medeiros, A. M. Z., da Silva, G. H., Brito, M. L., Faria, J. M. L., Delite, F. S., Paula, A. J., & Martinez, D. S. T. (2024). Biocorona Formation and Hemolytic Effects of Graphene Oxide-Silver Nanoparticles. *Journal of the Brazilian Chemical Society*, *35*(12). https://doi.org/10.21577/0103-5053.20240148

Ben Amor, A., Hemmami, H., Gherbi, M. T., Seghir, B. Ben, Zeghoud, S., Gharbi, A. H., Chenna, D., Ben Amor, I., Alsaeedi, H., Cornu, D., Bechelany, M., & Barhoum, A. (2025). Synthesis of spherical carbon nanoparticles from orange peel and their surface modification with chitosan: Evaluation of optical properties, biocompatibility, antioxidant and anti-hemolytic activity. *Biomass Conversion and Biorefinery*, *15*(7), 11345–11358. https://doi.org/10.1007/s13399-024-06291-w

Bernier, A., Tobias, T., Nguyen, H., Kumar, S., Smith, C. W., Sunasee, R., Ckless, K., Tuga, B., & Imtiaz, Y. (2021). Vascular and blood compatibility of engineered cationic cellulose nanocrystals in cell-based assays. *Nanomaterials*, *11*(8). https://doi.org/10.3390/nano11082072

Canham, P. B., & Burton, A. C. (1968). Distribution of size and shape in populations of normal human red cells. *Circulation Research*, *22*(3), 405–422. https://doi.org/10.1161/01.RES.22.3.405

Chahardoli, A., Qalekhani, F., Hajmomeni, P., Shokoohinia, Y., & Fattahi, A. (2025). Enhanced hemocompatibility, antimicrobial and anti-inflammatory properties of biomolecules stabilized AgNPs with cytotoxic effects on cancer cells. *Scientific Reports*, *15*(1). https://doi.org/10.1038/s41598-024-82349-z

Chan, H. W., Sheung, P. W., Tsao, S. T. M., Wu, C. Y., & Chuang, H. Y. (2025). Gold nanoparticle-loaded macrophages enhance radiotherapy via immune remodeling in oral cancer. *Materials Today Bio*, *33*. https://doi.org/10.1016/j.mtbio.2025.102029

Chaurasiya, S., M, V. M., Jangid, A. K., Medicherla, K., Pooja, D., & Kulhari, H. (2025). Synthesis and Evaluation of PEG Derivatized Nanostructures as Potential Delivery Carrier for Hydrophobic Drugs. *Nano Trends*, 100149. https://doi.org/10.1016/j.nwnano.2025.100149

Christodoulou, E., Tsimpolis, A., Theodorakis, K., Axypolitou, S., Tsamesidis, I., Kontonasaki, E., Pavlidou, E., & Bikiaris, D. N. (2025). Biodegradable Zwitterionic PLA-Based Nanoparticles:

Design and Evaluation for pH-Responsive Tumor-Targeted Drug Delivery. *Polymers*, *17*(18), 2495. https://doi.org/10.3390/polym17182495

Crawford, L., Wyatt, M., Bryers, J., & Ratner, B. (2021). Biocompatibility Evolves: Phenomenology to Toxicology to Regeneration. *Advanced Healthcare Materials*, *10*(11). https://doi.org/10.1002/adhm.202002153

de La Taille, T., Sarfati, P., Aid, R., Fournier, L., Pavon-Djavid, G., Chaubet, F., & Chauvierre, C. (2025). Microemulsion-Inspired Polysaccharide Nanoparticles for an Advanced Targeted Thrombolytic Treatment. *ACS Nano*, *19*(2), 2944–2960. https://doi.org/10.1021/acsnano.4c17049

De Menezes, Y. A. S., Félix-Silva, J., Da Silva-Júnior, A. A., Rebecchi, I. M. M., De Oliveira, A. S., Uchoa, A. F., & De F. Fernandes-Pedrosa, M. (2014). Protein-rich fraction of cnidoscolus urens (L.) arthur leaves: Enzymatic characterization and procoagulant and fibrinogenolytic activities. *Molecules*, *19*(3), 3552–3569. https://doi.org/10.3390/molecules19033552

Deng, H., Xu, M., Liu, H., & Gao, H. (2022). Clinical analysis of the characteristics of patients diagnosed with hemolytic anemia induced by hepatitis E viral infection. *ILIVER*, *1*(2), 117–121. https://doi.org/10.1016/j.iliver.2022.05.001

Dobrovolskaia, M. A., Clogston, J. D., Neun, B. W., Hall, J. B., Patri, A. K., & McNeil, S. E. (2008). Method for analysis of nanoparticle hemolytic properties in vitro. *Nano Letters*, *8*(8), 2180–2187. https://doi.org/10.1021/nl0805615

Dubach, I. L., Buzzi, R. M., Schaer, D. J., & Vallelian, F. (2025). Patterns of hemolysis, erythropoiesis, and iron distribution define unique disease trajectories in three mouse models of genetic anemia. *Experimental Hematology*, *147*. https://doi.org/10.1016/j.exphem.2025.104787

Dvanajscak, Z., Walker, P. D., Cossey, L. N., Messias, N. C., Boils, C. L., Kuperman, M. B., & Larsen, C. P. (2019). Hemolysis-associated hemoglobin cast nephropathy results from a range of clinicopathologic disorders. *Kidney International*, *96*(6), 1400–1407. https://doi.org/10.1016/j.kint.2019.08.026

Ebrahimi, S., & Bagchi, P. (2022). A computational study of red blood cell deformability effect on hemodynamic alteration in capillary vessel networks. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-08357-z

Huang, L., Guo, W., Zhao, T., Feng, Y., Li, Y., An, Q., Li, C., Tian, Y., Zhang, H., Zhou, C., Sun, Y., He, C., Niu, Z., Shen, H., & Xiang, B. (2025). Microfluidic fabrication of lipid nanoparticles for co-delivery of siRNA and hydroxychloroquine: An engineered theranostic platform for enhanced breast cancer treatment. *Chemical Engineering Journal*, *506*. https://doi.org/10.1016/j.cej.2025.160172

Ilhami, F. B., Puspitarini, S., Fitriana, Rahmawati, A., Mayasari, N. R., & Herliniati, H. (2025). Protective layer β-cyclodextrin within peanut (Arachis hypogaea L.) shells' nanoparticles enhances intracellular stable fluorescence for bioimaging applications: An in vitro and in silico study. *Plant Nano Biology*, *11*. https://doi.org/10.1016/j.plana.2025.100135

International Organization for Standardization (ISO). (n.d.). *ISO/TR 7406: Biological evaluation of medical devices—Guidance for hemocompatibility testing*.

Islamzada, E., Matthews, K., Lamoureux, E. S., Duffy, S. P., Scott, M. D., & Ma, H. (2022). Degradation of red blood cell deformability during cold storage in blood bags. *EJHaem*, *3*(1), 63–71. https://doi.org/10.1002/jha2.343

Jeong, J. H., Sugii, Y., Minamiyama, M., & Okamoto, K. (2006). Measurement of RBC deformation and velocity in capillaries in vivo. *Microvascular Research*, *71*(3), 212–217. https://doi.org/10.1016/j.mvr.2006.02.006

Kasprzyk, M., Opiła, G., Hinz, A., Stankiewicz, S., Bzowska, M., Wolski, K., Dulińska-Litewka, J., Przewoźnik, J., Kapusta, C., & Karewicz, A. (2025). Hyaluronic Acid-Coated SPIONs with Attached Folic Acid as Potential T2 MRI Contrasts for Anticancer Therapies. *ACS Applied Materials and Interfaces*, *17*(6), 9059–9073. https://doi.org/10.1021/acsami.4c20101

Kuck, L., Frappa, F. A., McNamee, A. P., & Simmonds, M. J. (2025). Storage temperature and anticoagulant choice determine the short-term stability of blood rheological parameters. *Clinical Hemorheology and Microcirculation*. https://doi.org/10.1177/13860291251376503

Luan, H., Peng, C., Yasin, P., Shang, Q., Xiang, W., & Song, X. (2025). Mannosamine-Engineered Nanoparticles for Precision Rifapentine Delivery to Macrophages: Advancing Targeted Therapy Against Mycobacterium Tuberculosis. *Drug Design, Development and Therapy*, *19*, 2081–2102. https://doi.org/10.2147/DDDT.S505682

Ma, L., Zhou, W., Ma, X., Li, X., Zhang, Q., Ma, W., Chen, N., Zhou, X. T., & Tang, X. H. (2025). Preparation and property evaluation of oral colon targeted protein delivery system with sodium alginate and chitosan. *Scientific Reports*, *15*(1). https://doi.org/10.1038/s41598-025-04983-5

Mayerhöfer, T. G., Pahlow, S., & Popp, J. (2020). The Bouguer-Beer-Lambert Law: Shining Light on the Obscure. *ChemPhysChem*, *21*(18), 2029–2046. https://doi.org/10.1002/cphc.202000464

Mazzarino, L., Loch-Neckel, G., dos Santos Bubniak, L., Ourique, F., Otsuka, I., Halila, S., Curi Pedrosa, R., Santos-Silva, M. C., Lemos-Senna, E., Curti Muniz, E., & Borsali, R. (2015). Nanoparticles Made From Xyloglucan-Block-Polycaprolactone Copolymers: Safety Assessment for Drug Delivery. *Toxicological Sciences*, *147*(1), 104–115. https://doi.org/10.1093/toxsci/kfv114

Medeiros, T. S., Bezerra de Lima, L. E., Alves-Pereira, E. L., Alves-Silva, M. F., Dourado, D., Fernandes-Pedrosa, M. de F., Figueiredo, R. C. B. Q. de, & da Silva-Junior, A. A. (2025). Cationic and anionic PLGA-cholesterol hybrid nanoparticles as promising platforms to enhance the trypanocidal efficacy of benznidazole and drug delivery in Trypanosoma cruzi-infected cells. *Biomedicine and Pharmacotherapy*, *183*. https://doi.org/10.1016/j.biopha.2024.117782

Na, R., Jing, J., Yang, H., Li, Y., Yuan, X., Sun, X., Han, J., Wang, J., Tong, Z., He, G., & Ye, W. (2025). Formulation and Evaluation of VCAM-1-Targeted Methotrexate Lipid Nanoparticles for Rheumatoid Arthritis Therapy. *International Journal of Nanomedicine*, *20*, 10977–10998. https://doi.org/10.2147/IJN.S532163

Neun, B. W., Ilinskaya, A. N., & Dobrovolskaia, M. A. (2018). Updated Method for In Vitro Analysis of Nanoparticle Hemolytic Properties. In *Scientia Pharmaceutica* (Vol. 79, Issue 3, pp. 91–102). https://doi.org/10.1007/978-1-4939-7352-1_9

Ngcamu, A. N., Nwabuife, J. C., Ndimande, N. P., Gabela, S., Ghazi, T., Sekhoacha, M. P., & Faya, M. A. (2025). Novel folic acid-functionalized smart Eicosapentaenoic acid liposomes for the targeted delivery of quercetin against cancer. *Journal of Drug Delivery Science and Technology*, *113*. https://doi.org/10.1016/j.jddst.2025.107358

Peng, C., Luan, H., Shang, Q., Xiang, W., Yasin, P., & Song, X. (2025). Mannosamine-Modified Poly(lactic-co-glycolic acid)-Polyethylene Glycol Nanoparticles for the Targeted Delivery of Rifapentine and Isoniazid in Tuberculosis Therapy. *Bioconjugate Chemistry*, *36*(5), 1021–1033. https://doi.org/10.1021/acs.bioconjchem.5c00062

Qian, H., Lv, J., & Hu, X. (2025). Development and evaluation of curcumin nano-niosomes for glioma-targeted therapy. *Scientific Reports*, *15*(1). https://doi.org/10.1038/s41598-025-95348-5

Rojas-Mancilla, E., Oyarce, A., Verdugo, V., Zheng, Z., & Ramírez-Tagle, R. (2015). The cluster [Re6Se8I6]3− induces low hemolysis of human erythrocytes in vitro: Protectives effect of albumin. *International Journal of Molecular Sciences*, *16*(1), 1728–1735. https://doi.org/10.3390/ijms16011728

Salimi, S., Motalleb, G., Dehghani, H., Rahdar, A., Dastjerdi, K., Velasco, B., & Taboada, P. (2025). Anticancer effect of tamoxifen and Fe3O4@SiO2@Cu hybrid NPs on malignant human breast cancer cell (MCF-7). *Journal of Molecular Liquids*, *429*. https://doi.org/10.1016/j.molliq.2025.127570

Shafique, S., Irshad, A., Ishtiaq, U., Mhamdi, L., Sharif, S., Jawad, R., Martin, P., M'Hamdi, N., Arshad, S., & Bejaoui, B. (2025). Reimagine breast cancer treatment: MgO-IDA nanocomposites: A promising strategy for precision medicine targeting MCF-7 cells. *Results in Chemistry*, *17*. https://doi.org/10.1016/j.rechem.2025.102660

Shakeel, V., Hussain Gul, I., John, P., & Bhatti, A. (2024). Biocompatible gelatin-coated ferrite nanoparticles: A magnetic approach to advanced drug delivery. *Saudi Pharmaceutical Journal*, *32*(6). https://doi.org/10.1016/j.jsps.2024.102066

Shakibaie, M., Hosseininasab, S. S., Riahi-Madvar, S., Adeli-Sardou, M., Jabari-Morouei, F., & Forootanfar, H. (2025). The potential of novel arsenic nanoparticles containing metformin (MTF@As NPs): a study on their antioxidant and cytotoxic properties. *BMC Chemistry*, *19*(1). https://doi.org/10.1186/s13065-025-01419-z

Sharifi, F., Ebrahimnejad, P., Mohammadi, H., Abasi, M., Asare-Addo, K., & Nokhodchi, A. (2025). Unlocking Curcumin's potential: Nano-encapsulation in mPEG-chitosan/hyaluronic acid complexes for enhanced therapeutic impact in colon cancer. *Journal of Drug Delivery Science and Technology*, *108*. https://doi.org/10.1016/j.jddst.2025.106894

Sherwood, L. (2016). *Human Physiology: From Cells tp Systems* (Ninth).

Shi, H., Wang, B., Shi, Z., Ma, H., Li, Y., Liu, Y., Zhao, Y., Xia, N., Wu, C., & Gao, Y. (2025). Paclitaxel-Ang-2-functionalized bionic mesoporous selenium nanoparticles for targeted therapy of glioma. *Pharmacological Research*, *216*. https://doi.org/10.1016/j.phrs.2025.107783

Sohail, S., Zeb, A., Alamri, A. H., Fatease, A. Al, Lahiq, A. A., Alruwaili, N. K., Khan, S., & Din, F. ud. (2025). Novel biocompatible multifunctional porous magnetic nanoclusters for the targeted delivery of lenvatinib towards hepatocellular carcinoma. *Materials Advances*, *6*(5), 1769–1787. https://doi.org/10.1039/d4ma01101e

Ullah, I., Kamal, A., Saba, M., Ara, U., Touhami, D., Wahab, A., Maqbool, T., Nazish, M., Alrefaei, A. F., & Lackner, M. (2025). Chlorophyllum molybdites-synthesized manganese oxide nanoparticles (MnO-NPs): morphology, biocompatibility, and anticancer properties against liver cancer (HepG2) cell line. *Scientific Reports*, *15*(1). https://doi.org/10.1038/s41598-025-93818-4

US FDA. (2023). *Use of International Standard ISO 10993-1, "Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process."* https://www.fda.gov/media/85865/download

Uyuklu, M., Cengiz, M., Ulker, P., Hever, T., Tripette, J., Connes, P., Nemeth, N., Meiselman, H. J., & Baskurt, O. K. (2009). Effects of storage duration and temperature of human blood on red cell deformability and aggregation. *Clinical Hemorheology and Microcirculation*, *41*(4), 269–278. https://doi.org/10.3233/CH-2009-1178

van Kampen, E. J., & Zijlstrat, W. G. (1983). Spectrophotometry of Hemoglobin and Hemoglobin Derivatives. In *Advances in Clinical Chemistry* (Vol. 23, pp. 199–257). Elsevier. https://doi.org/10.1016/S0065-2423(08)60401-1

Yaghoubi, A., Ramazani, A., Sillanpaa, M., Ghasemzadeh, H., & Mohammadi, E. (2025). Biocompatible porous PAM/CNT nanocomposite hydrogel films for sustained drug delivery and cancer therapy. *Scientific Reports*, *15*(1), 22387. https://doi.org/10.1038/s41598-025-05473-4

Yedgar, S., Barshtein, G., & Gural, A. (2022). Hemolytic Activity of Nanoparticles as a Marker of Their Hemocompatibility. In *Micromachines* (Vol. 13, Issue 12). MDPI. https://doi.org/10.3390/mi13122091

Zhaisanbayeva, B. A., Mun, E. A., Ulmanova, L., Zhunissova, Z., Umbayev, B., Olzhayev, F., Vorobjev, I. A., Hortelano, G., & Khutoryanskiy, V. V. (2024). In vitro and in vivo toxicity of thiolated and PEGylated organosilica nanoparticles. *International Journal of Pharmaceutics*, *652*, 123852. https://doi.org/10.1016/j.ijpharm.2024.123852

Zheng, C., Li, S., Mueller, J., Chen, C., Lyu, H., Yuan, G., Zamalloa, A., Adofina, L., Srinivasan, P., Menon, K., Heaton, N., Immenschuh, S., Silva, I., Rausch, V., Hammad, S., Dooley, S., Chokshi, S., Riva, A., He, S., & Mueller, S. (2025). Evidence for alcohol-mediated hemolysis and erythrophagocytosis. *Redox Biology*, *85*. https://doi.org/10.1016/j.redox.2025.103742