Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of pH-responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs

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Short Abstract:

A hemolysis assay is an early screen to gauge the ability of drug delivery systems to deliver their cargo into the cell cytosol. The assay measures the disruption of erythrocyte phospholipid membranes as a function of environmental pH.

Long Abstract:

Although there are many potential therapeutic targets inside the cell, the delivery of agents to address these intracellular targets poses a significant challenge. Frequently, drugs are internalized by cells and packaged into vesicles, only to become

shuttled back to the cell membrane via exocytosis, or degraded via the endosomal pathway.[1] In the latter process, the internal pH of the vesicles decreases to about 6.0, which is the optimal pH of enzymes that function in this compartment, such as lysozyme. Recently, polymeric nanoparticles have been engineered that take advantage of this phenomenon to facilitate cytosolic delivery of their cargo. These polymeric nanoparticles are zwitterionic and charge-neutral at physiologic pH (7.4), and do not interact with phospholipid membranes under these conditions. However, at pH 6.0 - 6.5, the polymers become protonated and acquire a net positive charge, increasing their ability to disrupt phospholipid bilayers, and therefore, lyse the endosomes containing the polymers. This activity has promoted the endosomal escape of peptide drugs and nucleic acids, allowing them to access their cytosolic targets.[2, 3]

Currently, the most reliable experimental model of this endosomolytic behavior is provided by the *ex vivo* hemolysis assay.[4] In this experiment, human red blood cells and polymeric nanoparticles are incubated in a series of buffers at defined pHs. Following a short centrifugation step to remove intact red blood cells, release of hemoglobin into the medium is measured by a spectrophotometer (405 nm for best dynamic range, but 450 or 541 nm are also acceptable). This is used as a surrogate measure for red blood cell lysis. This model has also been used by others to evaluate the endosomolytic behavior of cell-penetrating peptides and other gene delivery systems.[5, 6]

The hypothesis is that the polymers that result in the least hemolysis at pH 7.4, but significantly elevated hemolysis at pH < 6.5, will be the most effective candidates for cytosolic drug delivery. This is because polymers that fit these criteria would be expected to remain 'inert' until a drop in the local pH, which primarily occurs within endosomal compartments. Therefore, this environmental trigger is required for endosomal escape of the payload carried by the polymers.

In this protocol, erythrocytes will be isolated from a human donor, and co-incubated at pH 5.6, 6.2, 6.8, or 7.4 with experimental drug delivery agents. Any remaining erythrocytes are pelleted, and the supernatants (containing hemoglobin released from lysed erythrocytes) are analyzed via a plate reader (Figure 1).

Protocol Text:

1) Preparation and Sterilization of Buffers and Test Agents

1.1) 150 mM NaCl buffer: Dissolve 4.383 g NaCl crystals in 500 mL of nanopure water.

1.2) pH Buffers: Add concentrated HCl to bottles of D-PBS, dropwise with stirring, to adjust pH from 7.4 (as-purchased) to 5.6, 6.2, and 6.8. Check pH against several pH meters if possible to ensure accuracy of measurements.

1.3) Sterilize all buffers noted above through a bottle-top vacuum filtration apparatus.

1.4) 20% Triton X-100 (positive control): Mix 20 mL pure Triton X-100 in 80 mL of nanopure water. Shake vigorously and sonicate to dissolve. Leave at room temperature overnight before use.

2) Preparation of Erythrocytes

2.1) Obtain 25 ml of blood from anonymous human blood donor, stored temporarily in K_2-EDTA-coated Vacutainer tubes to discourage coagulation, according to IRB-approved Study Protocol.

2.2) Centrifuge blood at 1500 rpm for 5 minutes, and mark levels of blood (red, lower layer) and plasma (yellowish, upper layer) on tube.

2.3) Aspirate plasma gently via a micropipettor, and discard into bleach.

2.4) Fill to marked line (original level of plasma) with 150 mM NaCl solution. Cap and invert a few times to gently mix. Centrifuge at 1500 rpm for 5 minutes.

2.5) Repeat step 2.3-2.4 to wash blood cells again. Then aspirate supernatant and replace with PBS at pH 7.4. Invert to mix.

2.6) Split blood evenly into four tubes, corresponding to each pH that will be tested. Label the tubes according to each pH to be tested (5.6, 6.2, 6.8, 7.4).

2.7) Centrifuge blood tubes at 1500 rpm for 5 minutes. Mark levels on tubes, then aspirate supernatant.

2.8) Fill each tube to marked line with buffer of appropriate pH (as indicated in 2.6).

2.9) Label four 50 ml conical tubes (one per pH to test), and pipet 49 ml of PBS of appropriate pH into each conical tube.

2.10) Add 1ml of erythrocytes (same pH) into corresponding tube for a 1:50 dilution. Blood should be turbid and will settle upon sitting. If suspension appears clear, cells have lysed.

3) Lysis Assay Setup and Quantification

3.1) Prepare multiple stock solutions of all experimental agents, at 20x the desired final concentration to be tested (Assay will take 10µL of drug delivery agent + 190µL diluted red blood cells, leading to a 1/20 dilution of the original drug delivery agent into the final test mixture). Stocks of 20, 100, and 800 µg/mL are suggested, resulting in final concentrations of 1, 5, and 40 µg/mL, respectively.

3.2) Pipet 10 µL of each concentration of each experimental agent to a round-bottom 96-well plate. For optimal results, load each sample in triplicate or quadruplicate.

3.3) For positive control wells, add 10 µL of 20% Triton X-100 to some wells.

3.4) For negative control wells, add 10 µL of D-PBS at pH 7.4 to some wells.

3.5) Pipet 190 µL of diluted erythrocytes (see 2.10) to each well.

3.6) Incubate plates at 37°C for one hour (optional: Use an orbital shaker).

3.7) Centrifuge plates for 5 min at 1500 rpm to pellet intact erythrocytes.

3.8) Using a multichannel pipet, transfer 100 µL of supernatants to clear, flat-bottomed 96-well assay plates.

3.9) Measure absorbance of each well with a plate reader at 450 nm. If blood is too concentrated and high absorbance readings are obtained, plates can also be alternatively read at 541 nm. If blood is too dilute, and low absorbance readings are obtained, plates can also be alternatively read at 405 nm.

3.10) Using Microsoft Excel or some other plotting software, set absorbance of the negative control sample (step 3.4) to 0% hemolysis, and the absorbance of the positive control sample (step 3.3) to 100% hemolysis. After setting up a linear regression based on these two data points, use the resulting formula to calculate % hemolysis of each individual well.

Representative Results:

Typically, the agents that will correspond to the highest levels of cytosolic delivery of drugs, nucleic acids, or other bioactive molecules will exhibit pH-dependent hemolytic behavior. This is exemplified by Agent #1 as portrayed in Figure 2, which exhibits minimal hemolysis at pH 7.4, but a sharp increase in hemolytic behavior at endosomal pH ranges (< 6.5). Some agents that meet these criteria may exhibit considerable levels of hemolytic behavior at physiological pH ranges (Agent #2 at 40 µg/mL; Figure 2), suggesting that these agents may be undesirably toxic before arrival at a desired tissue of interest *in vivo*.

In most cases, hemolysis is also dose-dependent, as increasing concentrations of the nanoparticles correspond with higher levels of hemolysis, especially at the lower pH ranges tested (5.6 – 6.2).

Some agents, including detergents such as Triton X-100, which is used as a positive control, will be able to destabilize erythrocyte membranes regardless of pH. The results shown reflect this behavior as well.

Tables and Figures

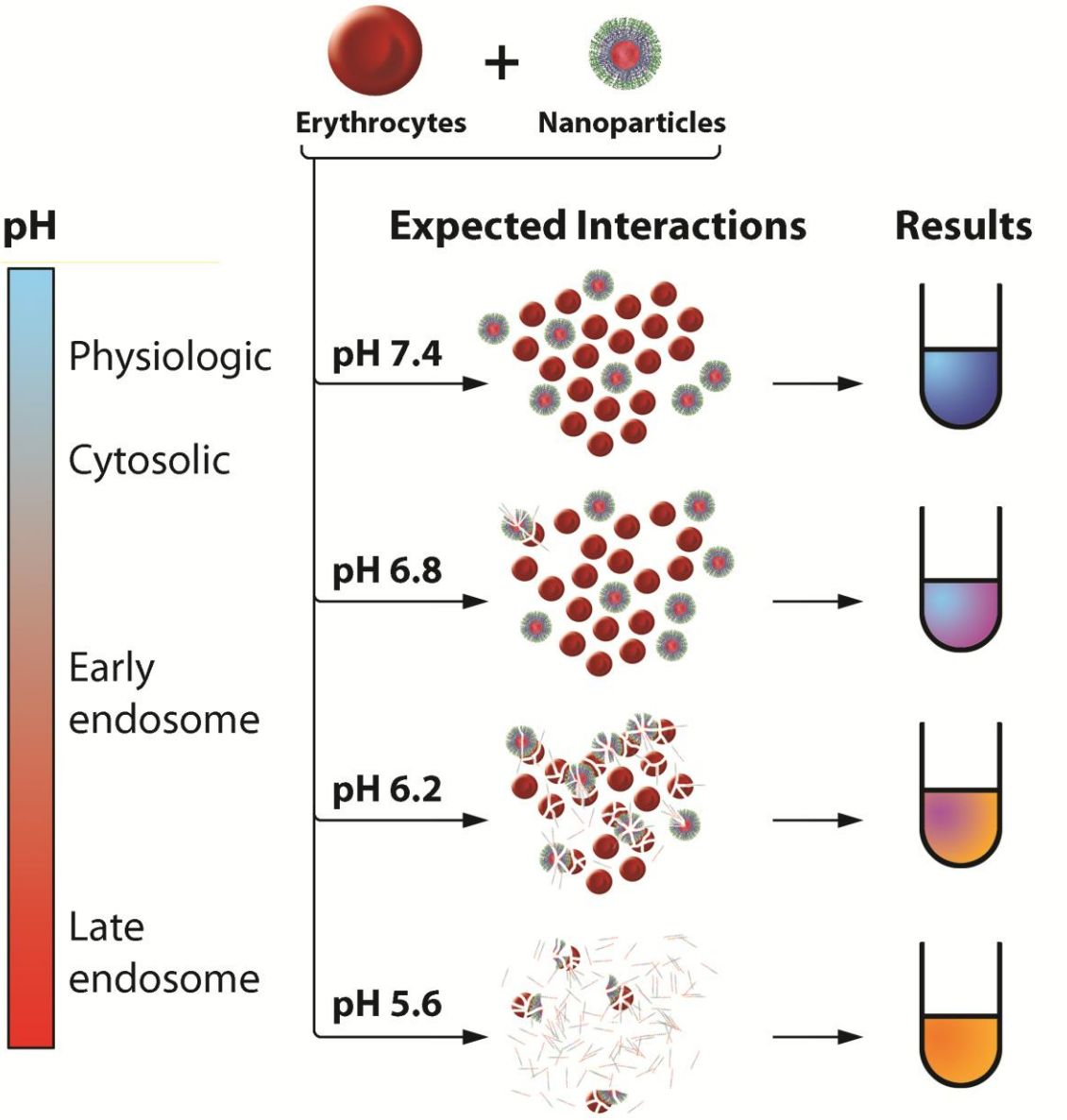

Figure 1. Schematic Diagram of Red Blood Cell Hemolysis Assay. Human erythrocytes are isolated and incubated with experimental drug delivery agents in a series of buffers simulating the pH range from physiologic (7.4) to late endosomes (5.6). The optimal drug delivery agents most likely to interact with late endosomes for cytosolic drug delivery will likely not interact with the erythrocytes at physiologic pH. However, at lower pH ranges, the agents are capable of destabilizing phospholipid membranes. The release of hemoglobin into the surrounding medium is measured via a plate reader.

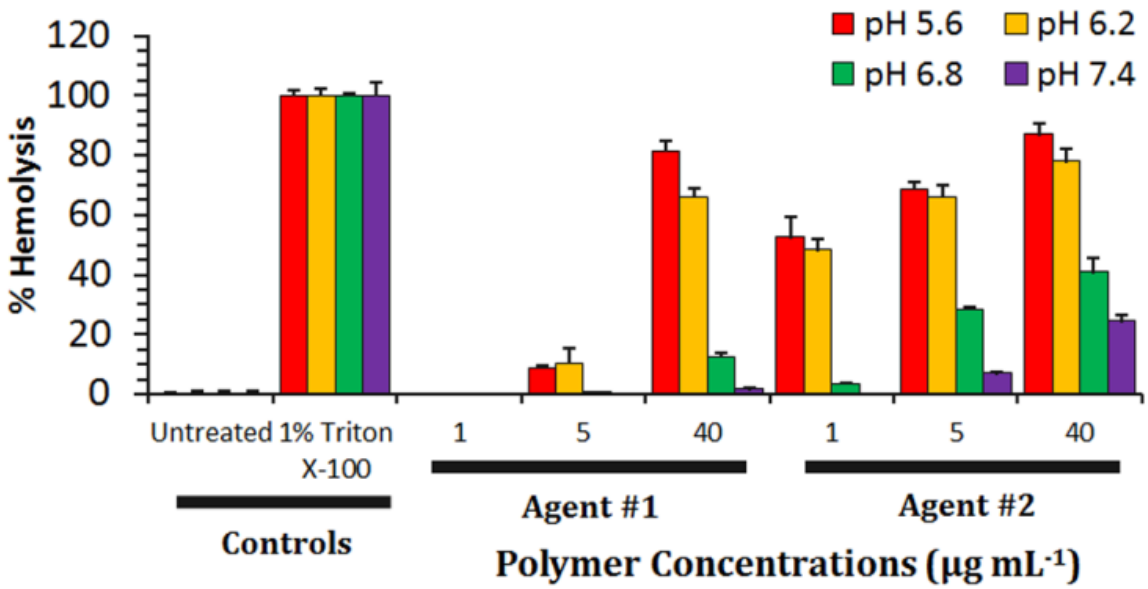

Figure 2. Representative Results of a Hemolysis Assay Demonstrating Behavior of Two Experimental Transfection Agents. A_{450} of the buffer incubated with untreated erythrocytes is set to 0% hemolysis, while A_{450} of buffer collected from Triton X-100-treated erythrocytes is set to 100% hemolysis. Based on these control samples, the ability of experimental transfection agents to lyse erythrocytes can be calculated. Typical agents exhibit dose-dependent hemolytic behavior, as well as pH-dependent hemolytic behavior. Ideal agents (such as Agent #1) exhibit minimal hemolysis at pH 7.4, but a sharp increase in hemolytic behavior at endosomal pH ranges (< 6.5). Some agents meet these criteria, yet exhibit substantial hemolytic behavior at physiological pH ranges (Agent #2 at 40 μg/mL), suggesting that these agents may be undesirably cytotoxic prior to arrival in endosomal compartments. Error bars indicate standard deviation of 4 independent measurements.

Discussion:

pH-responsive polymers that are capable of disrupting cellular membranes are capable of hemolysis of red blood cells at pH values encountered in the endosome (Figure 1; pH 6.8 – early endosome, pH 6.2 – late endosome, pH 5.6 – lysosome).[7-10] Furthermore, endosomal release of a variety of biomacromolecular therapeutics (e.g. peptides, siRNA, ODNs, proteins) has been correlated to red blood cell hemolysis in a variety of applications In previous studies, results from hemolysis assays have corresponded well with a polymer's performance as an intracellular drug delivery vehicle.[2-4, 11] Therefore, the pH-responsive, membrane destabilizing activity of polymeric drug delivery systems can be tested using hemolysis assays. This is an effective first screen to gauge the ability of polymeric drug carriers to mediate intracellular drug delivery based on their pH-dependent membrane interactions.

While the hemolysis assay utilizes the red blood cell membrane as a biological model for cellular membranes, the make-up and lipid content of endosomal membranes varies by cell-type and may not be accurately recapitulated by the blood cell membrane.[1] A variety of other assays have been developed to more accurately recapitulate endosomal entrapment and release *in vitro*.[12, 13] Many groups have synthesized liposomes containing fluorescence resonance energy transfer (FRET)-quenched fluorophores, which become unquenched following destabilization of the liposomes and release of the fluorophores to the surrounding media. In these studies, the quantification of unquenched fluorophores correlated with the ability of a vehicle to mediate endosomal escape of their payload.[12, 14] The most robust methods include microscopy-based measurements, including colocalization of fluorescently-labeled nanoparticles with dye-labeled lysosomes (e.g. LysoTracker by Life Technologies) or detection of intracellular trafficking of pH-sensitive dye-tagged polymers (e.g. pHrodo by Life Technologies).[15, 16] The advantage of the hemolysis assay over such methods is in its ability to screen drug delivery vehicles at a significantly higher throughput, in the presence of naturally-occurring biomembranes.

The use of this assay can also provide information on the mechanism through which a drug delivery vehicle can mediate endosomal escape. For example, gene delivery vehicles based on polyethyleneimine (PEI) lack an inherent ability to disrupt phospholipid membranes at neutral or acidic pH's.[6, 17] Instead, PEI achieves cytosolic gene delivery through a 'proton sponge' effect. After the internalization of PEI, it serves as a buffer in the endosome, soaking up acids introduced by the action of proton pumps on the endosomal membrane. Eventually, the buildup of ions inside the endosome results in a rise in osmotic pressure, leading to endosomolysis. Therefore, the success of proton sponge effect necessitates the incorporation of a critical concentration of PEI into an endosome.[17] Delivery vehicles that achieve endosomal disruption through the 'proton sponge' effect will not lyse red blood cells or liposomes, and their efficacy in achieving intracellular drug delivery must be assessed through osmotic pressure calculations or *in vitro* transfection studies.

The other two known, major physicochemical methods of mediating endosomal escape destabilize the phospholipid bilayer through the use of 'fusogenic' peptides or proteins (mediate membrane fusion), or the introduction of pores.[18] Drug delivery vehicles that mediate endosomal escape through either of these mechanisms have been shown to lyse erythrocytes, sometimes in a pH-dependent fashion.[4, 19]

In conclusion, the assay described here is a reliable model of the interactions between exogenous substances and the endosomal membrane. Because of the lack of confounding factors that may be present when using immortalized cell lines, this assay provides a high throughput means of drug delivery vehicle screening, enabling the rapid development of formulations that deliver biologics to interface with intracellular targets.

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Disclosures: IRB approval: Procedures involving human subjects have been approved by the Vanderbilt University Institutional Review Board (IRB; Protocol #111251). No conflicts of interest declared.

Table of specific reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
BD Vacutainer - K_2EDTA Vacutainer Tubes	Fisher Scientific	22-253-145	For blood collection
BD Vacutainer Blood Collection Needles, 20.5-gauge	Fisher Scientific	02-665-31	For blood collection
BD Vacutainer Tube Holder / Needle Adapter	Fisher Scientific	22-289-953	For blood collection
BD Brand Isopropyl Alcohol Swabs	Fisher Scientific	13-680-63	For blood collection
BD Vacutainer Latex-Free Tourniquet	Fisher Scientific	02-657-6	For blood collection
Hydrochloric acid (conc.)	Fisher Scientific	A144-500	For adjustment of pH of D-PBS.
Triton X-100	Sigma-Aldrich	T8787	Positive control
Dulbecco's PBS	Invitrogen	14190	
Nalgene MF75 Sterile Disposable Bottle-Top Filter Unit with SFCA Membrane	Fisher Scientific	09-740-44A	
BD 96-well plates, flat-bottomed, tissue culture-treated polystyrene	Fisher Scientific	08-772-2C	For plate-reading at the end of the assay.
BD 96-well plates, round-bottomed, tissue culture-treated polystyrene	Fisher Scientific	08-772-17	For incubation of red blood cells with experimental agents.

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