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Experimental and Analytical Improvement of Erythrocyte Function by LBL Self-Assembly

Zhiming Cao

Woodberry Forest School, 898 Woodberry Forest Road, Montford, VA 22960, USA
Tyler_Cao@woodberry.org

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Abstract: Red blood cells (RBCs) are ideal for research into drug delivery due to their high biocompatibility and ability to circulate throughout the human body. First, we isolated the RBCs and coated them with a silica film using tetraethyl orthosilicate (TMOS) and water. Then, we applied a polar polymer layer on top of the film and etched away the inner silica shell using a BOE solvent. This ensured that the remaining gel exhibited cell-like compliance, similar to that of human RBCs. This enabled the gel to deform while traversing the capillaries. Ultimately, we obtained a double-layered polymer membrane. We can use the characteristics of RBCs to attach drugs to the inner wall of the polymer layer. This model can treat systemic diseases with high targeting specificity and a long cycle. Further clinical trials are needed for future clinical applications.

1. Introduction

Due to the continuous growth of the global population and the rapid development of medical technology, the demand for blood resources in clinical settings has increased year after year. Currently, the blood supply relies heavily on volunteer donations. However, low donation rates and lengthy blood screening and preparation cycles have led to a chronic blood shortage. Additionally, whole blood and red blood cell products are perishable with limited preservation time, which makes long-term, large-scale stockpiling difficult. Thus, the blood shortage problem is particularly prominent in scenarios such as public health emergencies, large-scale disaster rescue, and battlefield first aid. Developing a safe, effective, and mass-producible blood substitute that can be preserved long-term has become an important issue in transfusion medicine and biomaterials.[1]

Red blood cells (RBCs) are one of the most important cellular components of blood and are responsible for key physiological functions, such as oxygen transportation and carbon dioxide excretion[2]. Their unique, biconcave, disc-shaped structure and high intracellular hemoglobin concentration give RBCs extremely high gas exchange efficiency. In addition to their oxygen-carrying function, red blood cells have been widely explored as natural drug delivery vehicles in recent years due to their excellent biocompatibility and long circulation time in vivo. Studies have shown that erythrocytes can evade recognition and clearance by the immune system, significantly prolonging the half-life of loaded drugs. Meanwhile, their internal hemoglobin and catalase can act synergistically to enhance the therapeutic effects of certain diseases. However, the limited availability of natural erythrocytes, their short in vitro storage time (usually no more than 42 days), and the risk of carrying bloodborne pathogens greatly limit their widespread use[3].

To overcome these limitations, researchers have developed functionalized artificial erythrocytes. The goal is to achieve large-scale, standardized production of erythrocyte substitutes and expand their applications in biomedicine. Layer-by-layer (LBL) self-assembly technology, a preparation method based on the alternating adsorption of oppositely charged polyelectrolytes, offers a novel approach to constructing artificial red blood cells. This technology's basic process includes wrapping a layer of silica (SiO_2) or a degradable polymer film around natural erythrocytes to create a template. Then, a multilayer polymer membrane is constructed on the template by alternately adsorbing positive and negative charges. Finally, the template is removed to produce erythrocyte-like carriers with flexible structures and functionalizable membrane layers. Ultimately, the natural erythrocyte membrane can be covered to enhance its immune escape ability[4].

This paper focuses on the application of layer-by-layer (LBL) self-assembly technology in preparing artificial erythrocytes, emphasizing the potential of this approach to integrate oxygen delivery, controlled drug release, magnetic targeting, and toxin detection. Through a systematic analysis of the advantages, challenges, and future directions of this technology, we aim to establish a theoretical foundation and technical reference for designing and translating the new generation of erythrocyte mimetic carriers for clinical use[5].

2. Methods and Materials

2.1 Materials

PBS solution, Ficoll (1.077 g/mL), RBC observation solution, blood cell plate, microscope, counter, 4% formaldehyde fixative solution, water, toluene, ice box, centrifuge, 4% paraformaldehyde solution, and RBC pellet mixture, centrifuge, BCA protein solution, working solution, a 96-well plate, 0.9% NaCl solution with pH = 3, TMOS, sterile tape, sodium dithionite, hemoglobin, alginate solution, chitosan solution, ethanol solution, BOE reagent and so on.

2.2 Methods

1) RBC Density Gradient Centrifugation Purification (Ficoll)

Mix 1 mL of PBS solution and 1 mL of blood sample in a test tube, then add 5 mL of Ficoll with a density of 1.077 g/mL and mix thoroughly. Place the mixture in a centrifuge and centrifuge at 20 ℃, 400g, 4 minutes increase, 0 minutes decrease 35 minutes.

After centrifugation, remove the supernatant and retain the remaining RBC precipitates. Add 10 mL of PBS solution and centrifuge at 4 ℃, 800g, 4 minutes, and 0 minutes for 10 minutes (repeat this operation three times after removing the supernatant). Add 20 mL of PBS solution and centrifuge at 4 ℃, 800g, 4 minutes, and 0 minutes for 10 minutes. (Repeat this procedure twice after removing the supernatant.)

2) Blood cell counting plate counting procedure

First, clean and disinfect the cover slip and hemocytometer with alcohol. Place the cover slip over the hemocytometer, then use a pipette to inject RBC observation solution into the hemocytometer from the edge of the cover slip. Place the hemocytometer under a microscope and use a counter to record the number of RBCs in the four corners and five large squares in the center of the hemocytometer.

3) Observation of natural RBC morphology and formaldehyde-fixed RBCs

Resuspend the RBC sediment in PBS solution. Add 10 μL of the processed RBCs with 990 μL of PBS solution—for observing cell morphology; prepare two tubes of 9 mL of 4% paraformaldehyde fixative solution, add 2 mL of the resuspended RBC sediment equally to each tube of 9 mL of 4%

paraformaldehyde, seal the mixed solution with a sealing membrane, and place it in a rotary incubator (20 rpm) overnight for 24 hours.

4) Method and steps for extracting and separating hemoglobin

Mix water and toluene in a 1:0.4 ratio to form a solution, stir thoroughly, and place in an ice box for 10–30 minutes. Add a small amount of PBS to the new blood sample, centrifuge for 5 minutes, remove the supernatant, add PBS again, centrifuge for 5 minutes, remove the supernatant again, and add PBS to the blood sample to a total volume of 1 mL. Add 1 mL of the mixture to the water and toluene mixture, place in an ice bath, and let it sit for 30 minutes. Remove the supernatant, use a needle to extract the red blood sample from the middle layer, and filter out the hemoglobin using a 0.2 μm membrane.

5) Washing, fixing RBCs, and morphological observation

After completing Experiment 2, remove the mixture and centrifuge at 25 ℃, 800g, 9 minutes, 9 seconds for 10 minutes. Remove the supernatant, add 9 mL of PBS solution, and continue centrifuging under the same conditions for 10 minutes (repeat this step twice). Remove the supernatant again, add 9 mL of 0.9% NaCl solution, and continue centrifuging for 10 minutes under the same conditions. Pour out the supernatant and resuspend in NaCl solution. Combine the two tubes to obtain a total volume of 1 mL of fixed RBCs. Adding 10 μL of fixed RBCs to 990 μL of 0.9% NaCl solution.

6) BCA quantitative detection of hemoglobin (including BCA standard curve determination) operational steps

Prepare BCA protein solution at 2 mg/mL. Dilute in steps with each concentration exceeding 25 μL, taking samples and retaining them. Dilute hemoglobin and take 10, 100, and 1000-fold dilutions, ensuring each well contains at least 30 μL of hemoglobin. Prepare a working solution at a 50:1 ratio and add 200 μL to each well of the 96-well plate. Shake for 10 seconds, then incubate at 37 ℃ for 30 minutes. Finally, measure the absorbance at 562 nm and calculate the protein concentration in each well. Observe the samples in step 5.

7) RBC mineralization process, TMOS mineralization solution preparation with water, enhanced mineralization process

Mix 50 mL of 0.9% NaCl solution with pH = 3 with 750 μL of TMOS, and let it stand for 1 hour for mineralization.

Add the fixed RBCs evenly to the settled liquid, and rotate at 20 rpm for 20 hours overnight. After removal, place in a 37 ℃ incubator for 4 hours for enhanced mineralization, then centrifuge for 5 minutes.

8) Method and steps for preparing PBS buffer solution and 0.9% NaCl (pH = 3) solution (experimental material preparation)

Preparation of PBS buffer solution: Pour the PBS solvent into a beaker, add water to dissolve, then transfer to a PBS solution bottle and top up to 2 L. Place the solution bottle in an ultrasonic mixer for thorough mixing, then dispense equal volumes into containers.Then preparation of 0.9% NaCl (pH=3) solution: Weigh 9g of NaCl into a beaker, add 1L of purified water, place in an ultrasonicator to mix, then pour into containers. Finally, seal both solutions with newspaper and sterile tape and place in a sterilizer for disinfection.

9) Preparation of self-assembling mother solution and diluent

Add 0.2 mg of chitosan to 20 mL of water. Add 0.2 mg of alginate to 20 mL of water. Dilute the resulting solution.

10) Preparation of drying eluent and gradient dehydration method for preparing dried RBCs

Prepare the solution according to the above proportions. Centrifuge the RBCs for 5 minutes after rotating for 20 hours, remove the supernatant, and dehydrate in a gradient from low to high (add solutions of different concentrations of EtOH, shake evenly for 10 minutes, then centrifuge (25 ℃, 3000 rpm, 5 minutes). Remove the supernatant and resuspend in EtOH to 1 mL.

11) Assembly and molding of artificial red blood cells and hemoglobin loading

Resuspend the RBCs, pour them into a drying dish, wrap the dish in aluminum foil with holes punched in it, and ventilate for 20 hours. Then, place the dish in a tube furnace for calcination. Prepare a solution of 300 μL of 100 mg/mL sodium dithionite. Place the oxygen-absorbing hemoglobin in a 96-well plate and add the sodium dithionite solution. Wash the calcined residue with 1 mL of ethanol in a 5 mL test tube. Centrifuge for five minutes at 25 ℃ and 3,000 RPM. Incubate with chitosan for one hour. Wash twice with ultra-pure water and resuspend with 1 mL of PBS solution. Use the strong corrosive properties of the BOE reagent to etch the internal silicon shell.

3. Experimental results and analysis

First, to obtain the double-concave, disc-like structure of red blood cells, the cells were purified from whole blood using density gradient centrifugation. Figure 1 shows the photoscopic image of the purified red blood cells. Next, to maintain the double-concave disc structure, the cells were fixed with polyformaldehyde (Figure 2). After fixation with paraformaldehyde, red blood cells lose their biological activity and retain only their shape and structural characteristics. They are also not easily destroyed by added reagents. Next, a tetramethylsilanic acid solution is added to the fixed red blood cells, surrounding all of their biomolecular interfaces with hydrogen-bonded interfacial water networks that exchange silicic acid precursors with water. These precursors are then amphoterically catalyzed to produce silica (Figure 3). Silica forms on the surface of red blood cells and has a double-concave disc structure. Next, the water is removed by gradient dehydration with ethanol. The dehydration rate is slowed as much as possible to keep the structure intact. Then, the sample is dried and calcined. Silica is resistant to high temperatures; however, the organic component is removed at 500 degrees Celsius, producing less carbon dioxide and water vapor. Figure 1c shows the pure silica template after calcination. The pure, double-concave, disc-shaped silica obtained (a replica of a red blood cell) is used as a template for layer-by-layer (LBL) self-assembly. This process involves alternately depositing the template in positively charged chitosan and negatively charged sodium alginate solutions. Electrostatic attraction binds the multilayers tightly. The result is a silica template coated with multiple layers of chitosan-sodium alginate. The presence of silica limits the deformability of the artificial red blood cells; therefore, the silica is removed by etching. Figure 4 shows the artificial red blood cells obtained. Then, to enable the artificial red blood cells to carry and supply oxygen, hemoglobin is adsorbed onto the chitosan via electrostatic action, and its oxygen-carrying and releasing capacity is measured. As shown in Figure 5-7, the absorption peak is near 410 nm during oxygen inhalation and reoxygenation. After oxygen release, the absorption peak shifts to 430 nm. This indicates that artificial red blood cells can carry and release oxygen.

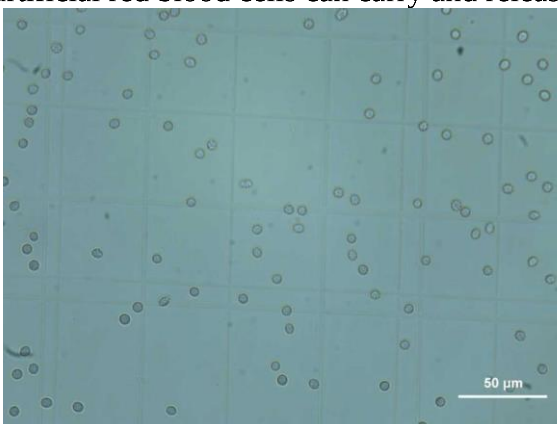

Figure 1 Natural RBC morphology

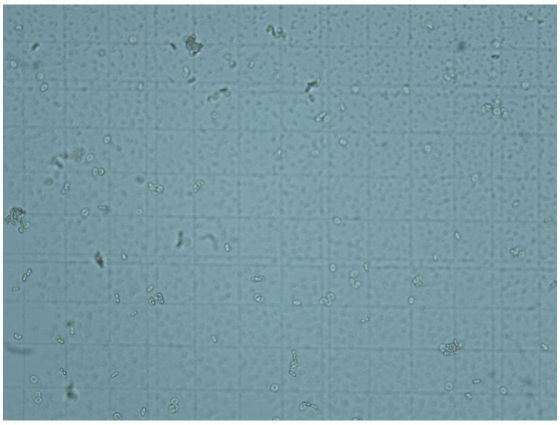

Figure 2 Fixed erythrocytes

Figure 3 High-temperature calcined polymer membrane

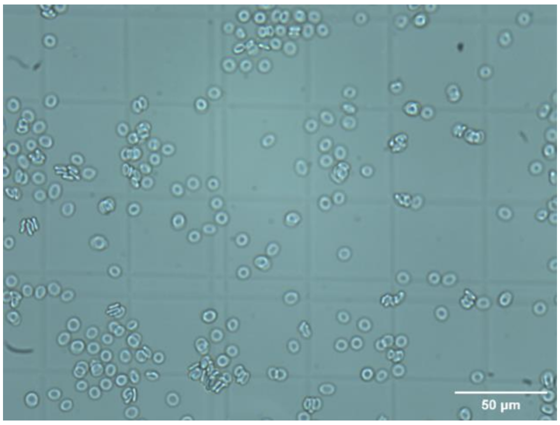

Figure 4 Artificial red blood cells

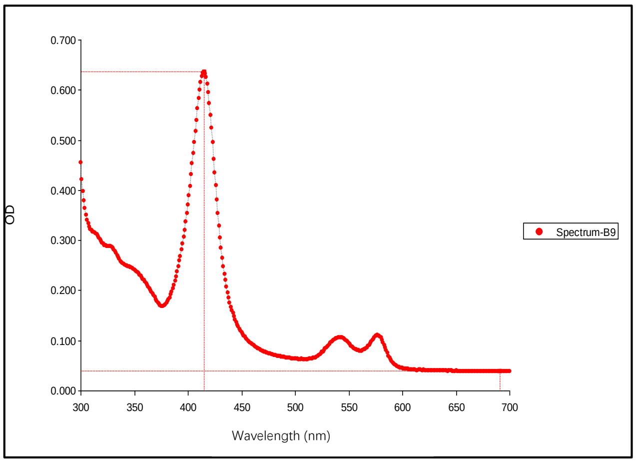

Figure 5 Oxygen uptake curve

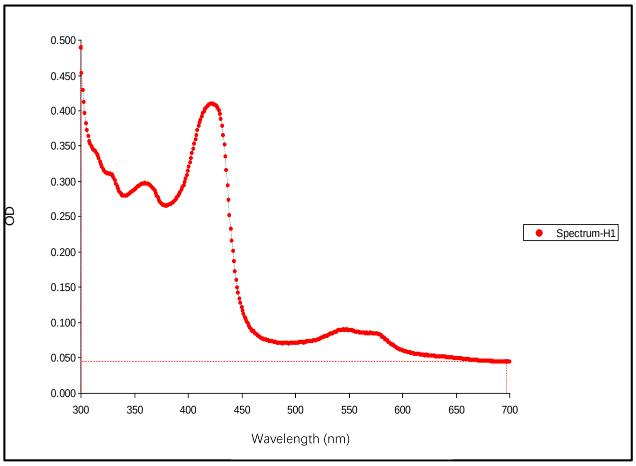

Figure 6 Oxygen release curve

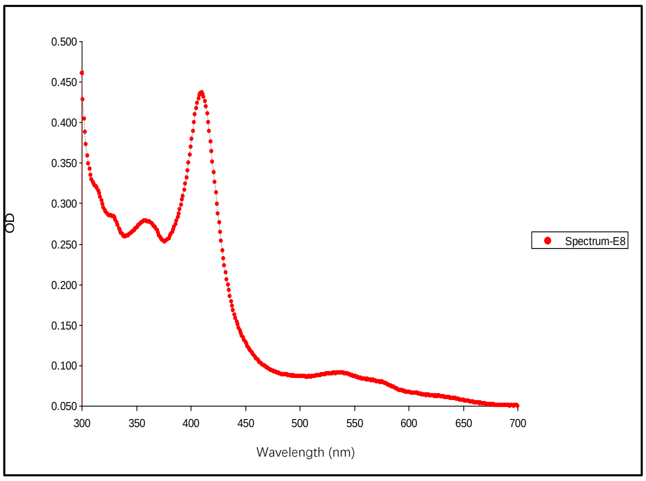

Figure 7 Reoxygenation curve

4. Discussion

This study focused on creating a polymer membrane on the exterior of red blood cells (RBCs) and proposed a method for making artificial RBCs that possess the characteristics of natural RBCs and enable additional new functions. These artificial red blood cells fully mimic natural RBCs in terms of their size, double-concave shape, deformability, oxygen-carrying capacity, and long circulation time. They possess typical deformability, zero hemolysis, low cytotoxicity, and good in vivo biocompatibility. They can also circulate in blood vessels for extended periods. Most importantly, it can carry various cargo to perform therapeutic drug delivery, magnetic targeting, and toxin detection.

Future work can explore the artificial RBC system's application in cancer treatment and in vivo toxin biosensing to expand its biomedical applications further. This study successfully replicated the complete RBC membrane structure and demonstrated the feasibility of artificial RBCs for drug transport. However, the slow preparation speed remains a significant challenge. Although artificial RBCs theoretically possess the functions and characteristics of red blood cells (RBCs) and can modify some of their functions, clinical trials and case studies are needed to validate this. Ideally, artificial RBCs would have a lifespan similar to that of normal RBCs, but this has yet to be proven. We can collect data through experiments and observations in the human body for further research. Drug delivery using RBCs is a long-term goal. We have currently established the basic framework, and after conducting clinical trials to evaluate its efficacy, it can be applied more widely. Based on human red blood cells (RBCs), we can also conduct experimental assembly using pig and cow RBCs. This technology could treat systemic diseases, such as gout, which require long-term drug storage in the body to circulate and treat various parts of the body.

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