ISSN 2959-409X

# Synthesis and exploration of the structure and function of artificially synthesized red blood cells

**Qiutong Fang**

Keystone Academy
11 Anfu Street, Houshayu'an Town, Shunyi District, Beijing
qiutong.fang@student.keystoneacademy.cn

**Abstract:**

Nowadays, many major diseases and surgical treatments require fresh blood to be injected into patients. However, fresh red blood cells' storage and typing conditions are very strict, which has led to research on artificial red blood cells. Through conducting research experiments on artificial red blood cells, human blood samples are subjected to a series of steps such as mineralization, filtration, and calcination, ultimately obtaining a small amount of artificial red blood cell samples. This series of research and learning has laid a solid foundation for the demand for large-scale blood production in the medical field, helping to improve the quality and quantity of blood resources in various medical facilities.

**Keywords: Synthesis,** Artificial Red Blood Cells, Hemoglobin, Oxygen absorption peak

## 1. Introduction

As the special connective tissue with various functions, blood is made of four different components, including plasma, white cells, platelets, and red blood cells. The red color of the blood is made of its key part, the red blood cells. 40% to 45% of blood is made of red blood cells; these cells are produced by the bone marrow and carry oxygen using a protein called hemoglobin to the whole body, starting from the lungs. There are two groups for hemoglobin: one is the heme group, which contains iron that contributes to the red blood cell's red color. The globin group is the protein that maintains and cries oxygen when the cell is transporting through the body. In contrast, red blood cells also exhale carbon dioxide and other wastes back to the lungs [1].

Nowadays, besides red blood cells naturally produced by people's bodies, artificial red blood cells are produced in different ways, including the separation and synthesis of hemoglobin, molecular modification, and artificial blood formula reconstitution. This kind of blood source can replace the original red blood cells for transporting oxygen to tissue and solve the blood shortage problem. [2]

The discovery of a replacement of blood started in 1616 when William Harvey found how human blood circulates inside the body. Doctors spent the following years discovering the replacement of milk, beer, and sheep blood. The first successful blood transfer was in 1667, but the patient died after the treatment. During the years of trying different replacements for blood, there were just limited materials that gained some success, such as milk. In 1854, the injection of milk was used to cure Asiatic cholera. In the 19th century, scientists first found a way of using hemoglobin and animal plasma as replacements for blood. In 1868, scientists found that using the solution of hemoglobin, which is isolated from red blood cells, can replace blood. 1871, scientists also found a way to use animal blood and plasma to replace human blood.

However, using the current techniques, isolating a large amount of hemoglobin is unachievable. Besides that, animal blood also contains some toxic elements that are toxic to humans. In 1883, by developing Ringer's solution, made of calcium salt, sodium, and potassium, scientists found it could cure the decrease of blood pressure caused by the loss of blood volume. But this still cannot play the role of artificial blood. Later, a scientist named Karl Landsteiner, the father of immunology, found the four blood groups. During WW1, people used a gum-saline solution made of galactose-gluconic acid to extend plasma. During the 1930s, the use of gum-saline solution diminished because of its negative health effects. During WW2, people found interest in studying blood and its replacement of it. Finally, the American Red Cross built the blood bank in 1947. In 1966, scientists used a new kind of chemical called perfluorochemicals (PFC) to replace blood. During the Vietnam conflict, people regained interest in finding hemoglobin solutions and other synthesized oxygen carriers. [3]

Past scientific studies on finding an ideal blood replacement have still not been able to achieve the goal of making human-made artificial red blood cells. Nowadays, scientists and red blood cell scholars use more advanced technologies and develop successful artificial blood by building a chitosan and sodium alginate structure on the calcined hemoglobin to attract oxygen ions and pass through human bodies.

The steps of this study are Ficoll purified blood, polyformaldehyde for fixation of red blood cells, hemoglobin extraction and measurement, mineralization and enhanced mineralization of hemoglobin, Gradient dehydration of hemoglobin using anhydrous ethanol and HMDS, Constructing Sodium Alginate and Chitosan Structures on Hemoglobin. This study aims to successfully create a sample of artificial red. Blood cells. The contribution of this study is to further develop the skill of creating artificial red blood cells and to create additional blood sources.

## 2. Materials and Methods

### 2.1 Materials(See Table 1 for details)

**Table 1 Consumables, instruments, and their specifications and brands**

| Material/ instrument name | Size | brand |
|---|---|---|
| Test tubes | 5ml/15ml/50ml | SAINING |
| Pipettes | 10μl/20μl/100μl/1ml | CORNING |
| Hettich ® MIKRO 220/220R Centrifuges | / | Sigma-Aldrich |
| PBS solution | 500ml | Gibco™ |
| Ficoll solution | 50ml | Sigma-Aldrich |
| Hemoglobin | 5g | Macklin |
| Pipettor | 10μl/20μl/100μl/1ml | BRAND |
| toluene | 100ml | Macklin |
| syringes | 20ml | D&B |
| filters | 0.22μm | Merck millipore |
| sodium alginate | 100g | Macklin |
| chitosan | 100g | Macklin |
| acetic acid | 50ml | Macklin |
| pure water | 4.5L | 哇哈哈 |
| BCA protein quantitative kit | / | Servicebio |
| ethanol | / | Macklin |
| optical microscope | / | XTALQUEST |
| ELIASA | / | Leopard |
| Ficoll | 25g | HARVEYBIO |
| pasteur pipet | 5ml/10ml | BKMAM |
| microscope slide | / | Sigma-Aldrich |
| coverslip | / | Servicebio |
| pH meter | / | SALMART |
| TMDSO | 100g | Strem |
| Shaker | / | Servicebio |
| BOE | 1L | Sigma-Aldrich |

### 2.2 Methods

#### 2.2.1 Experiment 1: Ficoll Purified Blood

1) Transfer 1ml of whole blood into a 15ml centrifuge tube, dilute with 10ml of PBS solution, and gently mix well.

2) Take two 15ml centrifuge tubes and add 5ml Ficoll solution first. Then, gently add the diluted blood to the upper layer of the Ficoll in two centrifuge tubes, being gentle to avoid mixing the two solutions. Dilute 10 ml of blood in each centrifuge tube.
3) 800g, 20 minutes; note that the deceleration setting must be set to no break or only 10-20% braking. After centrifugation, layers will be obtained, as shown in the figure.
4) The cell layer where RBC is located is red. The cells in that layer can be aspirated into another clean 15ml centrifuge tube using a straw.
5) Add PBS to 10-15ml, 800g, centrifuge for 10 minutes, remove the supernatant, and then add culture medium for the same cleaning process, repeating 5 times.
6) Add 5-10ml PBS to resuspend cells and store at 4 ℃.

**2.2.2 Experiment 2: Paraformaldehyde immobilizes red blood cells**

1）First, prepare the blood red blood cell solution that has been centrifuged and separated the previous day, and use a 1000ul pipette to draw blood from the red blood cells. Add an equal proportion of PBS to dilute the red blood cells precipitated at the bottom of the bottle, and mix well with a pipette. Take 10ul of the mixed solution and prepare to observe its morphology. Prepare a microscope observation pool and glass slides, disinfect with alcohol, wipe dry, and set aside. Carefully squeeze the diluted red blood cells along the lower edge of the glass slide into the central depression of the cross-shaped observation pool.
2）Clamp the collected specimen pool in the center of the optical microscope objective, adjust the brightness, and focus to observe the total number of cells in the upper left corner, upper right corner, middle, lower left corner, and lower right corner. The total number of cells is obtained by using the cell counting formula:
3) Observe the status and quality of red blood cells during the process to see if they are in a double concave shape.
4) Add 15ml of 4% paraformaldehyde to the red blood cell solution for fixation, and rotate on a rotary apparatus at room temperature for 24 hours.

**2.2.3 Experiment 3: 20 hours of mineralization and 3 hours of enhanced mineralization**

1) Remove the fixed red blood cell solution from the rotator.
2) Place red blood cells containing polyformaldehyde fixative into a high-speed centrifuge once for ten minutes at 4 ℃, 800g.
3) Use a plastic straw to remove the floating polyformaldehyde on the surface of red blood cells, add PBS solution, and rinse well. Place the centrifuge again for five minutes, keeping the temperature and speed unchanged.
4) Use a plastic straw to remove the floating PBS on the surface of red blood cells, add PBS solution, and rinse well. Place the centrifuge again for five minutes, keeping the temperature and speed unchanged.
5) Use a plastic straw to remove the floating PBS on the surface of red blood cells, add PBS solution, and rinse well. Place the centrifuge again for five minutes, keeping the temperature and speed unchanged.
6) Add 175ul of tetramethylsiloxane (TMOS) to 50ml of 0.9% NaCl (pH=3) and hydrolyze for one hour to form a mineralized solution.
7) Add hydrogen chloride gradually to the pH value of 15ml NaCl and adjust it to a pH value of approximately 3. Adding hydrogen chloride during the process resulted in a low pH value, which was adjusted by adding a small amount of sodium oxide.
8) Use a plastic straw to remove the floating PBS on the surface of red blood cells, add 2ml of 0.9% NaCl, and rinse well to precipitate. Put it back into the prepared TMOS, mix well, and mineralize on a rotary instrument for 20 hours. (Pay attention to whether the gas column in the test tube on the rotator flows normally and is obstructed).
9) To prepare the reserve solution, 2g of chitosan and 2g of sodium alginate are added to two tubes of 20ml ultra-pure water, respectively. Subsequently, 200ul of acetic acid is added to the chitosan test tube to accelerate melting.
10) Accelerate mixing chitosan and sodium alginate using an ultrasound and mediation instrument.

**2.2.4 Experiment 4: Anhydrous Ethanol and HMDS Gradient Dehydration**

1) Place the test tube containing hemoglobin on a shaking table at 37 ℃ for 2-3 hours for enhanced mineralization.
2) After mineralization, take 10ul and observe its morphology on a blood cell counting plate under an optical microscope.
3) Take three groups of 200ul of blood and add 25ul of PBS solution to each group, totaling 225ul, and place them in an enzyme-linked immunosorbent assay kit to test the blood oxygen uptake curve.
4) Deoxygenate hemoglobin with sodium hydrosulfite and then inhale oxygen. Mix three groups of 150ul hemoglobin and three groups of 50ul sodium hydrosulfite into three samples. If successful oxygen inhalation is not possible, it indicates poor cell quality.
5) Calculate the ratio of blood red blood cell ETOH to HMDS solution after cleaning and mineralization, a total of 8 tubes.
6) Add the prepared eight tubes of solution to the miner-

alized red blood cells in sequence, one tube at a time. Mix the mixture on a rotary apparatus for ten minutes, then set it to 4 ℃ on a centrifuge and centrifuge for 5 minutes at 3000RPM.

**2.2.5 Experiment 5: Vacuum drying and calcination of red blood cells**

1) Prepare the eighth tube of ETOH and red blood cell solution after centrifugation the previous day, and suck out the surface floating material. Wash the remaining precipitated red blood cells with 1 ml of anhydrous ethanol.
2) Put the solution into a clean calcination dish, add it to a vacuum-drying oven for 30 minutes, and perform dehydration at room temperature.
3) After dehydration, add it to a tube furnace and heat it to 500 degrees Celsius for 30-60 minutes before calcining for 4 hours.
4) During the calcination process, measure the oxygen uptake curve and release curve of hemoglobin using an enzyme-linked immunosorbent assay (ELISA) reader, then measure the oxygen uptake curve. The oxygen absorption curve uses three tubes of 990ul PBS+10ul Hb. The oxygen release curve was obtained using three tubes of 900ul PBD+100ul Hb and one tube of a solution made of 100mg sodium bisulfite and 1000ul PBS (100ul each placed in three tubes). Finally, an air pump adds deoxygenated hemoglobin to the three tubes, inhaling oxygen for 5-6 minutes each.
5) After the red blood cells are calcined, let them cool and remove them. Rinse the red blood cells in the calcined dish with 2ml of ethanol and use an ultrasound to prevent clumping. A 10ul pipette transfers red blood cells to the observation pool, and then the morphology of red blood cells is observed using an optical microscope.

**2.2.6 Experiment 6: Coating of Chitosan and Sodium Alginate and Etching silica**

1) Centrifuge the precipitate of calcined Erythrocyte template in anhydrous ethanol for 5 minutes at a temperature of 4 ℃ and a speed of 3000 rpm.
2) Absorb anhydrous ethanol from the surface of the Erythrocyte template.
3) Set aside the previously prepared chitosan and sodium alginate for later use.
4) Dilute 10mg/ml of chitosan 5 times, take 2ml and add it to a 10ml tube, then add 8ml of ultrapure water for 10ml.
5) Dilute 10mg/ml of sodium alginate 10 times, take 1ml and place it in a 10ml tube, add 9ml of ultrapure water, for a total of 10ml.
6) Wash the calcined Erythrocyte template well with 500ul of ultrapure water, and add 500ul of chitosan diluted 5-fold.
7) Place the rotating frame and mix for 1 hour. Centrifuge at 20000rcf for 10 minutes at 4 ℃. Remove the supernatant and rinse well with 500ul of ultrapure water.
8) Add 500ul of sodium alginate diluted 10 times, take 500ul each, and rinse well with a pipette.
9) Place the rotating frame and mix for 1 hour. Centrifuge at 20000rcf for 10 minutes at 4 ℃. Remove the supernatant.
10) Repeat steps 6 to 9 three times.
11) The coating product of 500μL LBL was mixed with 500μLBOE solution and etched for 1min.
12) Full wash with ultra-pure water, 20000rcf, 10min and repeat washing three times.

**2.2.7 Experiment7: Extraction and Concentration Determination of Hemoglobin**

1) Freeze fresh blood in an ice box and let it stand still.
2) Extraction solution preparation: Take 7ml of pure water and 2.8ml of toluene solution, mix well in a 10ml centrifuge tube, freeze, and let stand in an ice box for layering.
3) Add 1ml of fresh blood to the extraction solution, mix well, and let stand on an ice box for 30 minutes until hemoglobin and membrane lipids separate. Use a plastic straw to seal the film grease along the tube wall. Afterward, a 0.22um filter with a syringe is assembled to filter the membrane lipids and retain the hemoglobin. Mix 50ul of hemoglobin with 50ul of PBS solution to create three wells, add 96 well plates respectively, and place the plates in an enzyme-linked immunosorbent assay (ELISA) reader for oxygen absorption peak detection.
4) Detect the 540nm hemoglobin porphyrin ring and substitute the results into the formula（, ）.
5) Configure low-permeability PBS and 0.9% NaCl.
6) Measure the prepared 15ml NaCl solution using a pH tester; the result is pH 4.5.

**2.2.8 Experiment 8: Hemoglobin loading**

1)Take 500μl of washed chitosan - sodium alginate and add 500μl of chitosan.
**1)** Place the rotating frame and mix for 1 hour. Centrifuge at 20000rcf for 10 minutes at 4 ℃. Remove the supernatant and rinse well with 500ul of ultrapure water.
3) Add 500μl2mg/ml hemoglobin at 4℃ for 24h.
4) Repeat steps 1 to 3 three times.
5)Add 500μl sodium alginate**,** place the rotating frame, and mix for 1 hour. Centrifuge at 20000rcf for 10 minutes at 4 ℃. Remove the supernatant and rinse well with 500ul of ultrapure water.

# 3. Results

First, to obtain the double-concave disc-like structure of red blood cells, red blood cells were purified from whole

blood by density gradient centrifugation. The photoscopic image of purified red blood cells is shown in Figure 1a. Subsequently, to maintain the double-concave disc structure of the red blood cells, the red blood cells were fixed with polyformaldehyde. After fixation with paraformaldehyde, the red blood cells lose their biological activity, retain only their shape and structural characteristics, and are not easily destroyed by the added reagents[4]. Then, tetramethylsilanic acid solution was added to the fixed red blood cells, and all of the biomolecular interfaces of the RBC were surrounded by hydrogen-bonded interfacial water networks that exchange silicic acid precursors with water. These precursors are then amphoterically catalyzed to produce. Silica is formed on the surface of the red blood cells and has a double-concave disc structure[5] (Figure 1b). The water is then removed by gradient dehydration with ethanol, and the dehydration rate is slowed as much as possible to keep the structure intact, then dried and calcined. Silica is resistant to high temperatures, while the organic component will be removed at 500 degrees Celsius as less carbon dioxide and water vapor. The pure silica template after calcination is shown in Figure 1c. The obtained pure double-concave disc-shaped silica (a replica of a red blood cell) is used as a template for LBL self-assembly, which is alternately deposited in a positively charged chitosan solution and a negatively charged sodium alginate solution, and electrostatic attraction is used to bind the multilayers tightly[6]. Currently, a silica template coated with multiple layers of chitosan-sodium alginate is obtained. The presence of silica limits the deformation ability of artificial red blood cells, so silica is removed by etching. Then, to make the artificial red blood cells able to carry and supply oxygen, the hemoglobin is adsorbed on the chitosan by electrostatic action(Figure 1d), and its oxygen-carrying and oxygen-releasing capacity is measured. As shown in Figure 2, during oxygen inhalation and re-oxygen inhalation, the absorption peak is near 410nm, while after oxygen release, the absorption peak is near 430nm, which indicates that the artificial red blood cell can carry and release oxygen.

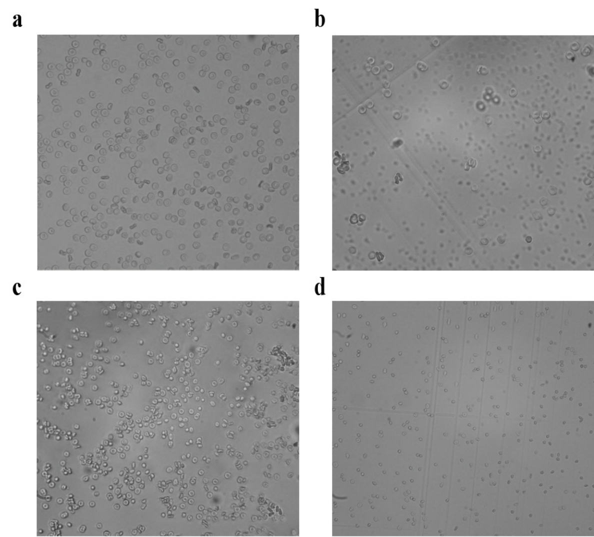


**Figure 1 a) Light microscope image of normal red blood cell; b) Light microscope image of a mineralized silica template; c) Optical microscope image of a calcined silica template; d) Photoscopic image of the final artificial red blood cell containing hemoglobin.**

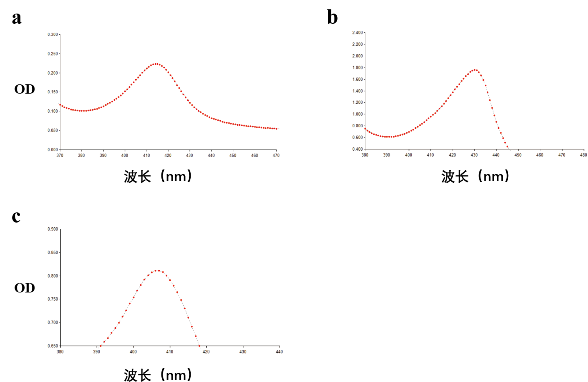


**Figure 2 a) Oxygen uptake curve of artificial red blood cells; b) Oxygen release curve of artificial red blood cells; c) Artificial red blood cell reoxygenation curve.**

## 4. Discussion

This article briefly studies the background and production methods of artificial blood red blood cells, as well as the analysis of the peak values of hemoglobin oxygen uptake, release, and reabsorption during the process, through three parts. The article also explores the importance of the production process of artificial blood cells. The main function of artificial red blood cells is to replace the abundant and demanding natural red blood cells in our department

as much as possible and to assist medical needs such as surgical treatment in hospitals. During the experiment, the production of artificial red blood cells was practiced. The experiments were divided into Ficoll purification of blood, polyformaldehyde fixation of red blood cells, extraction and determination of hemoglobin, mineralization enhancement of red blood cells, gradient dehydration of red blood cells by anhydrous ethanol and HMDS, vacuum drying and calcination of red blood cells, and construction of oxygen-carrying chitosan and sodium alginate frameworks on red blood cells. Ultimately, an ideal prototype of artificial red blood cells was produced. During the experiment, the enzyme-linked immunosorbent assay (ELISA) was used multiple times to proficiently operate hemoglobin and conduct deeper research. The oxygen absorption, oxygen release, reabsorption, and porphyrin ring of hemoglobin were detected, and data were collected. The limitation of this study is that due to the imprecise operation during calcination, the samples were slightly damaged under microscope observation, resulting in a limited number of available samples. Secondly, due to the short research time cycle, the chitosan and sodium alginate framework was not encapsulated multiple times, and human hematopoietic red blood cells were not successfully oxygenated. This cannot produce a complete and usable artificial red blood cell.

The significance of this study mainly lies in the preliminary practice of immediate artificial red blood cell technology, attempting to restore a relatively complete process of artificial blood cell production.

Due to the increasing prevalence of chronic diseases, there is an increasing demand for blood transfusions. [7] Meanwhile, people are increasingly aware of the risks associated with traditional blood transfusions, including infections and immune responses, which further drive the demand for artificial blood substitutes. [8] This has expanded the market for artificial blood. The artificial blood substitute market in 2023 is expected to reach $8.09 billion and is expected to reach $35.31 billion by 2031, with a compound annual growth rate of 20.20%. [9] In my opinion, the potential research direction for artificial blood in the future is to produce red blood cells on a large scale and with higher efficiency to meet more blood needs more quickly.

## 5. Conclusion

In this study, synthetic red blood cells were explored. Through layers of self-assembly of chitosan and sodium alginate on the silica template, the skeleton of synthetic red blood cells was successfully prepared. The hemoglobin was loaded on the skeleton of synthetic red blood cells by electrostatic adsorption, and the oxygen-carrying function of artificial red blood cells was finally realized.

## References

[1] Guo, J.; Agola, J. O.; Serda, R.; Franco, S.; Lei, Q.; Wang, L.; Minster, J.; Croissant, J. G.; Butler, K. S.; Zhu, W.; Brinker, C. J. Biomimetic Rebuilding of Multifunctional Red Blood Cells: Modular Design Using Functional Components. ACS Nano 2020, 14 (7), 7847–7859. https://doi.org/10.1021/acsnano.9b08714.

[2] Mount Sinai Health System, Department of Pathology and Laboratory Medicine, Icahn School of Medicine, New York, NY, USA; Khan, F.; Singh, K.; Mount Sinai Health System, Department of Pathology and Laboratory Medicine, Icahn School of Medicine, New York, NY, USA; Friedman, M. T.; Mount Sinai Health System, Department of Pathology and Laboratory Medicine, Icahn School of Medicine, New York, NY, USA. Artificial Blood: The History and Current Perspectives of Blood Substitutes. Discoveries 2020, 8 (1), e104. https://doi.org/10.15190/d.2020.1.

[3] Sarkar S. Artificial blood. Indian J Crit Care Med. 2008 Jul;12(3):140-4. doi: 10.4103/0972-5229.43685. PMID: 19742251; PMCID: PMC2738310.

[4] Hobro, A. J.; Smith, N. I. An Evaluation of Fixation Methods: Spatial and Compositional Cellular Changes Observed by Raman Imaging. Vib. Spectrosc. 2017, 91, 31–45. https://doi.org/10.1016/j.vibspec.2016.10.012.

[5] Meyer, K. C.; Coker, E. N.; Bolintineanu, D. S.; Kaehr, B. Mechanically Encoded Cellular Shapes for Synthesis of Anisotropic Mesoporous Particles. J. Am. Chem. Soc. 2014, 136 (38), 13138–13141. https://doi.org/10.1021/ja506718z.

[6] Bozuyuk, U.; Yasa, O.; Yasa, I. C.; Ceylan, H.; Kizilel, S.; Sitti, M. Light-Triggered Drug Release from 3D-Printed Magnetic Chitosan Microswimmers. ACS Nano 2018, 12 (9), 9617–9625. https://doi.org/10.1021/acsnano.8b05997.

[7] Moradi, S.; Jahanian-Najafabadi, A.; Roudkenar, M. H. Artificial Blood Substitutes: First Steps on the Long Route to Clinical Utility. Clin. Med. Insights Blood Disord. 2016, 9, CMBD.S38461. https://doi.org/10.4137/CMBD.S38461.

[8] Haldar, R.; Gupta, D.; Chitranshi, S.; Singh, M. K.; Sachan, S. Artificial Blood: A Futuristic Dimension of Modern Day Transfusion Sciences. Cardiovasc. Hematol. Agents Med. Chem. 2019, 17 (1), 11–16. https://doi.org/10.2174/1871525717666190617120045.

[9] Patil, Kajal.2023. Artificial Blood Substitutes Market Size, Share and Forecast 2031.linkedin.12-8.https://www.linkedin.com/pulse/artificial-blood-substitutes-market-size-share-forecast-kajal-patil-ohnnf .