Encapsulation of Streptavidin labeled with Alexa Fluor 750 into Erythrocytes

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Abstract

Erythrocytes, Red Blood Cells (RBCs), are known as oxygen carriers in a body. However, RBC's have also been utilized as biocompatible bioreactor and drug delivery vehicles. There are several methods that permit appropriate yield drug encapsulation into erythrocytes. The hypo-osmotic dialysis method is the most commonly employed technique, which is based on a high-hematocrit dialysis procedure. The use of RBCs as carriers for fluorescence labeled biosensors has not been exploited. Thus, we have been investigating the feasibility of utilizing RBCs as carriers for biocompatible fluorescent sensors. We hypothesized that labeled proteins could be successfully loaded into RBCs and fluorescence can be obtained. Streptavidin labeled with Alexa Fluor 750 (SA-AF 750) was encapsulated into RBCs via the hypo-osmotic dialysis method. The loaded cells were then scanned by using a spectrofluorometer. The results displayed a peak signal at 775 nm, which was an indication of presence of AF 750 in the RBCs. The protocols developed here could be utilized in the future to design *in vivo* optical sensors.

Author Keywords: erythrocytes, proteins, fluorescence, Alexa Fluor 750

1. Introduction

Erythrocytes, Red Blood Cells (RBC's), function as oxygen/CO_2 transporters. Their biconcave disk shape promotes and facilitates maximum transport. There are approximately 5 million RBCs/microliter of blood in circulation (totaling 30 trillion in humans), with lifetimes of 120 days [1]. Erythrocytes have remarkable capacity to undergo reversible membrane swelling. This flexibility in the membrane allows large pores to open and thus allow metabolites and macromolecules to cross the membrane [2].

For more than 20 years the properties of RBCs have been carefully investigated by a number of different researchers and laboratories for use as storage containers (erythrocyte encapsulation) for drug delivery [3,4]. Additionally, encapsulated erythrocytes have been proposed as carriers and bioreactors for the treatment of various diseases [5-12]. Erythrocytes are attractive systems due to their ability to deliver proteins and therapeutic peptides. They have been used also as transport for enzymes, such as L-asparaginase, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AlDH) among others, for the correction of metabolic alterations.

There are several methods currently in use that allow the encapsulation of agents into erythrocytes. These methods are based on a high-hematocrit dialysis procedure [7]. The procedure for the encapsulation of RBCs entails placing washed RBCs in dialysis tubing together with the substance to be encapsulated. The suspension is then dialyzed against a hypotonic solution until the erythrocytes have been lysed. The dialyzed cells, once in equilibrium with the exogenous substance, are resealed by restoring the physiological isotonicity, then washed and resuspended in plasma or in a physiological saline solution.

Utilizing RBCs as carriers for biosensors has not been investigated. As a first step in the development of a RBC-based biosensor, we examined the loading of a fluorescent dye within RBCs. We utilized Alexa Fluor 750 as the fluorophore to be encapsulated. Then we excited the fluorophore and examined its emission in phosphate buffer saline as well as through porcine aortas.

Alexa Fluor 750 dye with a weight of approximately 1100 Dalton is a long wavelength dye. Its fluorescence emission maximum is at 779 nm, which is well separated from commonly used far-red fluorophores, facilitating multicolor analysis. With a peak excitation at approximately 752 nm, conjugates of the Alexa Fluor 750 dye can be excited by a xenonarc lamp or dye-pumped lasers [13].

This paper will focus on:

1. The hypo-osmotic technique to load long emitting fluorophores into red cells in order to demonstrate signal capture through red cells.
2. Signal acquisition through porcine aorta of long emitting fluorophores mixed with red cells to demonstrate signal capture through tissue.

The results from this work will be used in the design of optical sensors encapsulated in RBCs.

2. Materials

The following materials were used in the experiment:

10,000 MWCO slide-a-lyzer cassettes (Cole-Parmer – EW-02905-42)
20G needles; 3ml syringes
Phosphate buffer, pH 7.4 500ml:

1. 202.5ml 200mM Na_2HPO_4 (81mM final)
2. 47.5ml 200mM Na_2HPO_4 (19mM final)
3. 250ml H_2O

Dialysis buffer 1L:

1. 250ml phosphate buffer
2. 36.4ml 110mM $MgCl_2$
3. 713.6ml H_2O

Streptavidin-Alexa Fluor 750 1100 MW, 1mg (Molecular Probes – A-20011)
DMSO 78.13 MW (MU Recycling – D-5879 – lot 100F-0269)
PBS 0.01M, pH 7.4 (Sigma - P-3813 - lot 075K8206)
500ml BV WH Blood NS Heprin (Pal-Freez Arkansas – 37132-1)

3. Instrumentations

Beckman centrifuge was used to spin down and purify the RBCs from plasma, platelets, and white cells. A scanning fluorescence spectrometer (FluoroMax-3 Jobin Yvon, Hobira) was used to collect fluorescence emission spectra by exciting the sample at 749 nm. The slit size and integration time were 4 nm and 0.5 s, respectively.

4. Method

There are several methods for the encapsulation of macromolecules into erythrocytes [6,8,14]. Most of these procedures involve the dialysis of erythrocytes against a hypotonic solution, the addition of the substance to be encapsulated and the resealing of the lysed cells. Other methods are also available (e.g. electroporation, drug-induced endocytosis and the osmotic pulse method) but usually these are best suited for the processing of very small volumes of erythrocytes. The hypo-osmotic dialysis method we have used is based on the following steps:

1. Washed red blood cells (bovine) were mixed with an isotonic solution containing isotonic NaCl and the relevant dyes. In the experiment, the suspension contained 90% cells and 10% dye solution.
2. Two mls of the mixture was placed in a Pierce dialysis slide (MW cut off ~10,000). The slide was placed in a beaker of ice-cold solution containing 4 mM MgCl2, 25 mM Na phosphate, pH 7.4. The incubation time was 30 minutes.
3. During this time, the red blood cells lyse and the contents equilibrate with the solution inside the slide (hemoglobin and dye mix) and the small molecules escape out of the dialysis slide.
4. The dialysis slide was then transferred to a beaker with ice cold solution of 165 mM NaCl, incubated for 30 more minutes on ice during which time the NaCl enters the slide and the cells.
5. Then the slide was transferred to 200 mls of an ice cold solution containing 150 mM NaCl, 10 mM Pi (Na salt) pH 7.4, 5 mM adenosine, 5 mM glucose, 5 mM $MgCl_2$ and incubated an additional 30 minutes.
6. Then the cells were incubated at 37C for 1 hour to reseal.
7. The red blood cells were then removed from the dialysis slide and washed twice in 165 mM NaCl by centrifuging for 3 minutes at 10,000 rpm. (Note that red blood cells do not need to be in a buffered solution because of the high buffer capacity of hemoglobin and the robust pH equilibration via the anion exchanger).

After encapsulation, the red cell solution was placed in a 1.26 mm (inner diameter) tygon tubing. A special holder was machined to hold the tube in an ISA spectrofluorometer. The loaded cells were scanned using the spectrometer. Figure 1 displays the set-up with the porcine tissue wrapped around the curvette.

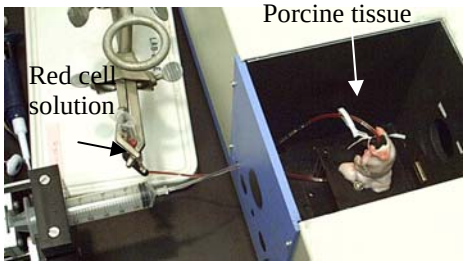

Figure 1. Set up of the tissue wrapped.

5. Results and Discussion

The fluorescence spectra of the cell pellets were determined. Figure 2 displays fluorescence spectra of the cells loaded with AF750. Since hemoglobin absorbance at ~800 nm is 200 liter/mol·cm, and AF750 is 250,000 liter/mol·cm, AF750 has ~1000 times greater absorption. Thus we see a strong fluorescence peak at ~770 nm. The volume of the dye in the red cells was ~10 µm.

Figure 3 shows the fluorescence of the loaded RBCs with different probes concentrations through porcine tissue. At a loading concentration of10uM, the peak signal was around 9700 cps, but when the concentration of the probes

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was dropped to 1uM, the peak signal was dropped to 2000 cps. These results showed that 1) red blood cells can be successfully loaded with fluorescence dyes; 2) it is possible to capture fluorescence through porcine tissue and 3) the loading concentration will play an important role in the success of capturing fluorescence.

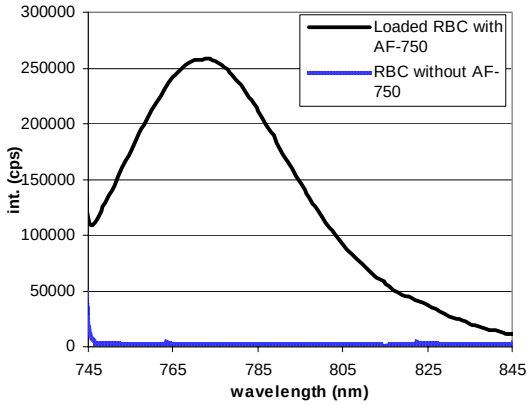

Figure 2. Fluorescence signal acquisition of AF750 dye loaded into red cells.

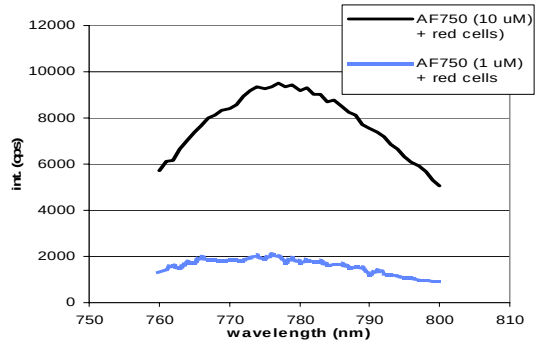

Figure 3. Fluorescence signal capture of AF750 dye through a porcine aorta.

6. Conclusion

The objective of this study was to demonstrate the ability to capture fluorescence from fluorophore-encapsulated red cells. We have utilized the hypo-osmotic dialysis technique to successfully make "ghosts" that were loaded with the fluorophore, AF750. This finding could be used in developing optical sensors for detecting several analytes, such as glucose. Currently, we are developing a glucose sensor using Fluorescence Resonance Energy Transfer (FRET) that will allow *in vivo* glucose sensing without biocompatibility and biofouling problems. These sensors may eventually be deployed for *in vivo* glucose sensing with superior sensitivity.

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