SPATIAL AND TEMPORAL DELIVERY OF SMALL MOLECULES FROM LONG WAVELENGTH SENSITIVE PHOTOTHERAPEUTCS USING ERYTHROCYTES AS BIOLOGICAL CARRIERS

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CHAPTER 5: LIGHT-MEDIATED DRUG THERAPY AND CELL CARRIERS: A CHAPTER FOR NON-SCIENTISTS

5.1. What's in your Medicine Cabinet?

Have you ever grabbed a cold remedy or prescription drug from the pharmacy knowing full well that this treatment may result in unintended side effects? Side effects result from medicine attacking healthy parts of the body instead of just the problem areas. The more powerful the medicine, the more intense and life-threatening those effects tend to be. For instance, while seasonal allergy sufferers often experience medicine-induced drowsiness, doctors are actually forced to restrict life-saving chemotherapeutics from cancer patients because the side effects become life-threatening. Researchers in the drug discovery and delivery fields are developing systems that target drugs directly to disease sites so that treatment spares healthy parts of the body and patients experience fewer unintended symptoms.

Researchers are exploring more effective ways to deliver drugs to disease sites. Many approaches involve attaching drugs to synthetic drug carriers that better accumulate at disease sites. Unfortunately, medical providers often have concerns over introducing foreign material into the body. For almost a decade, our lab has instead focused on using vitamins, in combination with carriers derived from human cells, as a means to deliver drugs in a targeted fashion. I will first describe the two main parts of our unique drug delivery system: 1. vitamin B12 (B12) as a light-controlled launching pad to regulate the time and space of drug delivery and 2. red blood cells (RBCs), which safely carry B12-drug-based launching pads through the body.

5.2. The Light-Induced Chemical Launching Pad

Before application to drug delivery was even a thought, our lab developed a unique light system to study biological processes in human cells, taking advantage of B12 photochemistry (light chemistry). While many people are familiar with B12 as a diet supplement, a lesser known property of B12 is its interaction with and subsequent response to light. My lab realized they could take advantage of this sensitivity to create a light-induced drug release system. Let's look at the molecule more closely (Figure 5.1) while we discuss its important functionalities for drug delivery.

The three parts of B12 we will look at more closely are the Co-C bond (a cobalt metal bound to a carbon atom, Figure 5.1, blue arrow), the corrin ring (a large carbon-based ring that is the "core" of vitamin B12, Figure 5.1, green arrow), and a 5′-OH (a highly reactive chemical group, Figure 5.1, red arrow).

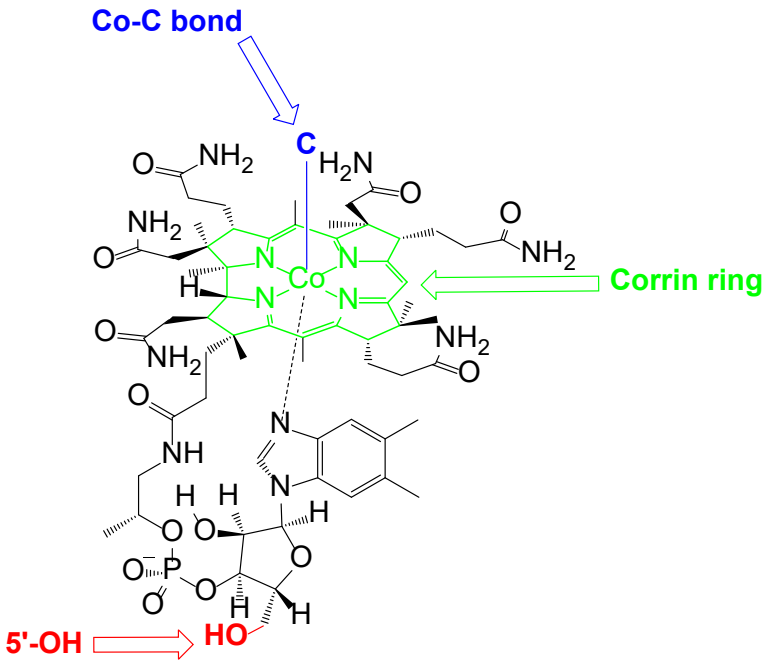

Figure 5.1. Vitamin B12. Highlighted groups are important for light-induced drug release. We substitute the C in the Co-C bond (blue) with drugs and other small molecules, which can be released on command by illuminating the molecule with low energy light. The corrin ring (green) absorbs light, which breaks the Co-C bond. The 5′-OH (red) is not affected by light and can be substituted with molecular antennas to increase light absorption.

The Co-C bond holds drugs to vitamin B12 like a grappling hook that we can release on command. Normally, the Co holds a molecule that the body naturally breaks off and uses. However, we can easily substitute that C with a drug or other chemical of interest. Out of all the bonds on vitamin B12, why this one? Chemical bonds are usually very strong, but the Co-C link is weak enough to break with a low energy source, such as colored light.

The corrin ring naturally captures green light (550 nm) of medium energy. We believe the green energy results in excited electrons that destabilize the Co-C bond, causing it to fall apart. This photochemistry has been understood since the 1970s. However, useful applications of this property have been limited…until now.

We discovered that when the C is replaced with a light-absorbing molecule called a fluorophore, we can speed up the bond breakage. Essentially, the fluorophore acts like a TV or radio antenna to amplify the light capturing effect. While this novel discovery was very exciting, we knew that it would have limited usefulness in the human body in its current state. You can see why if you've ever placed laser pointers of different colors under your fingertip. Green laser pointers do not penetrate human tissue very well because green light is absorbed and scattered as it travels through the body. By contrast, red lasers *do* penetrate the skin. When you place a green and then a red light against your finger, you can see the difference (Figure 5.2). The wavelength range (600 - 1000 nm) that best penetrates skin is collectively called the "optimal window of tissue penetration". In order to create a biologically useful system, we realized we could attach fluorophore antennas to B12, which would force it to respond to red light instead of green, which takes us into the third part of the molecule, the 5′-OH.

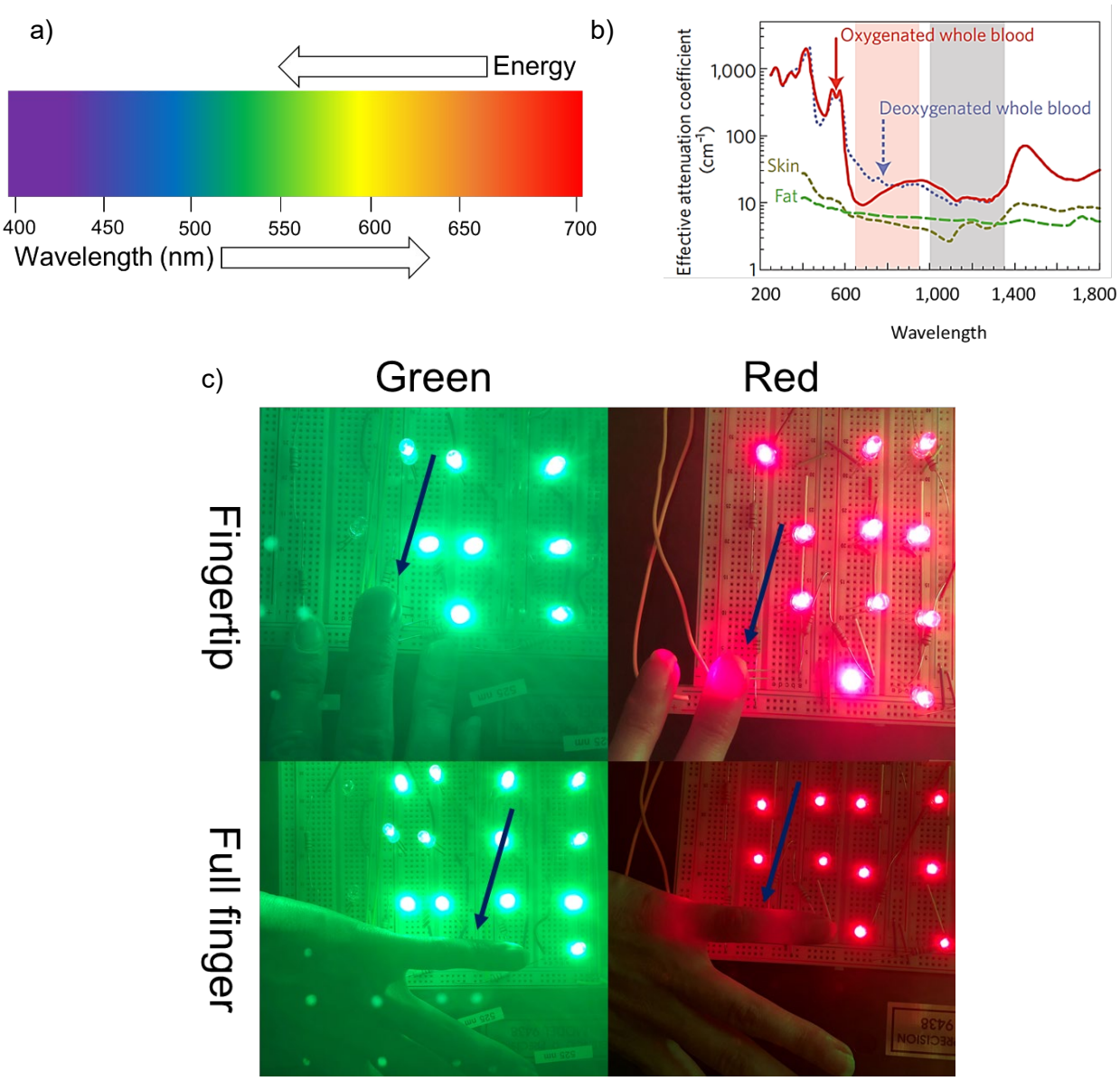

Figure 5.2. Visible light penetration. a) The visible light spectrum. Short wavelengths near the blue end have high energy while long wavelengths near the red end have low energy. b) Blood, skin, and fat strongly absorb light. This absorption is minimalized between 600 - 1000 nm. c) Light penetration of household LEDs through a human finger. Left: Biological species in the body easily prevent green light from penetrating human tissue, such as a fingertip or small finger. Right: Alternatively, red light in the “optimal window of tissue penetration” transverses human tissues.

We can fasten red light absorbing antennas to the 5′-OH group on the bottom of the corrin ring. With antennas, B12 strongly absorbs red light and releases drugs attached to the Co-C bond. Until our work began, light-activated drug release systems only responded to blue or green light or else required incredibly high-intensity light sources. Our antenna technique represents the first of its kind to use simple light sources, such as laser pointers, to release cargo with biologically useful red light.

In summary, we used chemical modification techniques to design a launching pad for the light-controlled release of various drugs within the optical window of tissue penetration. Figure 5.3 illustrates a fully constructed B12 phototherapeutic, Cy5-B12-Paclitaxel. Paclitaxel is a common chemotherapeutic. Imagine administering Cy5-B12-Paclitaxel and illuminating only the tumor site so that drug is only delivered to that area. Light could be as simple as LEDs or as complicated as fiber optic cables, depending on the need. While initial clinical studies would be done in a hospital, we eventually imagine a system where after injection, a patient could self-dose at home with a specially designed laser (Figure 5.4).

Such a direct, non-invasive delivery method has the potential to reduce common side effects associated with chemotherapy such as allergic reactions, muscle pains, and heart problems by treating only the tumor site.

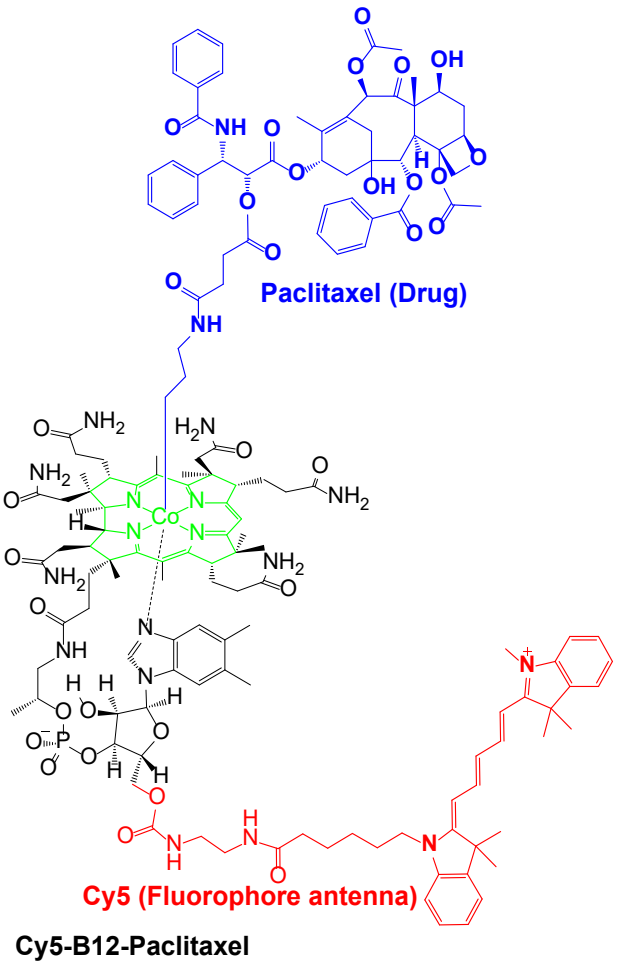

Figure 5.3. Cy5-B12-Paclitaxel. An example of an antenna-B12-Drug conjugate. Cy5 serves as a long wavelength antenna with the ability to capture red light and use that light energy to release the chemotherapeutic drug, Paclitaxel on command.

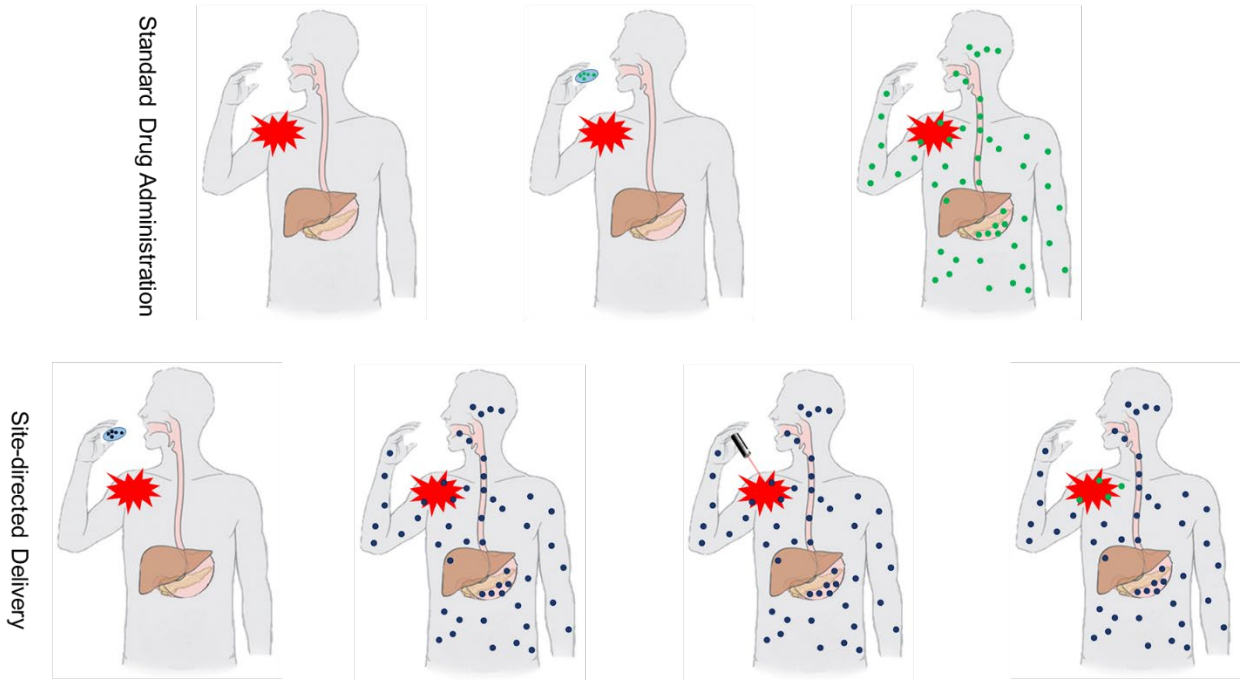

Figure 5.4. Site-directed drug delivery. Top: Often, we take medication to treat a very specific symptom, such as shoulder pain. We usually take medicine either orally (shown) or though injection. Our circulation system carries active ingredients all over our body. Medication is now damaging not only the site of the symptom, but also healthy areas, such as our stomach. Bottom: Site-directed delivery has the potential to alleviate side effects caused by off-target action. The ingredient that is spread all over our body is now inactive, not damaging healthy tissue it comes in contact with. External activation, such as with a light source, would treat only the symptomatic area.

5.3. Repurposing RBCs as Drug Carriers

As mentioned before, synthetic drug carriers are non-ideal for clinical use due to concerns with administering unnatural materials into the body. Some popular nanomaterials have been clinically approved, but scientists still struggle to create more targeted drugs that are safe, effective, and long-lasting. With this challenge in mind, we investigated carriers derived from patient's own cells to carry our phototherapeutics. We determined that RBCs possess the flexibility, storage capacity, safety, and circulation time that we desired in a drug carrier. In the 1970s, much research was conducted to load and use RBCs as drug carriers. However, as HIV/AIDS became more widespread in the 1980s, many researchers turned away from using blood products in favor of "safer" synthetic materials. Since the 1990s, a resurgence of interest in RBC carriers has led to the formation of two companies and the investment in automated equipment that loads various therapeutics into RBCs.

With our light-activated launching pad and cell carrier in hand, we set out to combine these two strategies and create a fully functional drug delivery system. My graduate research touched on three generations of RBC-B12 drug delivery: membrane decoration, internal encapsulation, and a design for rodent models with first pre-clinical investigations.

5.4. Generation 1: Membrane Decoration and Anti-inflammatories

Most synthetic drug carriers act like balls covered with Velcro, to which drugs can be attached on the outside. We used this idea to design the first generation of B12-loaded RBC carriers in which lipid anchors (fats composed of long chains of carbon) hold B12-drugs and antennas on the outside membrane. This strategy is technologically the easiest of all the loading strategies since lipids naturally stick to membranes when the two are mixed together. Thus, it was an ideal system to test light-mediated drug release from cells for the first time.

Human blood is surprisingly easy to purchase in a lab from companies who contract out to blood banks. By spinning blood (FedEx overnight!) in a centrifuge for 10 minutes, we separate RBCs from other blood components (plasma, white blood cells, and water). Then, we attach lipid anchors to B12-drugs and to antennas and create the final system by simply mixing the RBCs with the lipidated materials (Figure 5.5).

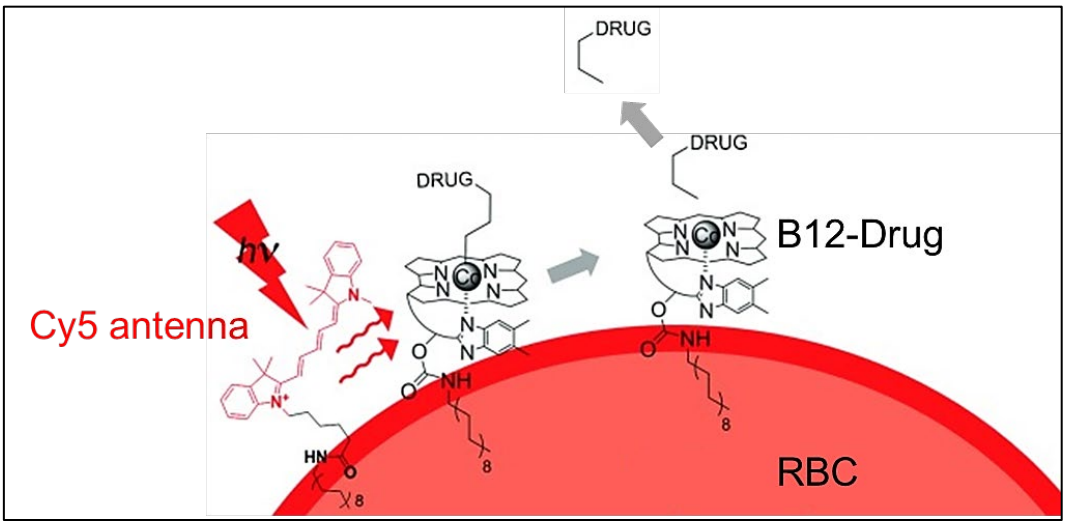

Figure 5.5. Attaching drugs to membranes. We attach lipidated antennas and lipidated B12-drugs to RBC membranes through simple mixing and incubation. The antennas absorb red light and transfer electrons to the B12-drug, breaking the Co-drug bond and releasing the drug.

Which drugs did we test with our system? For the first generation, we chose an array of anti-inflammatories with potential value in arthritis treatment (methotrexate, dexamethasone, and colchicine). Our light system would be an advantageous alternative to frequent injections of arthritis drugs, many of which also cause side effects. How would an arthritis phototherapeutic work? Since the drug is encapsulated into RBCs, we imagine using standard blood transfusion techniques already approved. Then, light treatment could be administered directly to the joint, moderating side effects. Natural RBCs circulate for up to 120 days, and our hope is to achieve a similar lifetime with loaded RBCs, reducing the need for more frequent injections.

5.5. Generation 2: Internal Encapsulation and Extension to Chemotherapeutics

With our first generation drug delivery system in hand, we recognized that membrane loading comes with some challenges. The most notable that, unlike a lab dish, proteins and other cell membranes also circulate in the human body and can pull off anchored material from the RBC membrane. While creating a stronger anchor is one approach, we also recognized that we could tap a different part of the RBC: the cell interior.

The RBC interior is unique in ways that make it especially advantageous as a drug carrier over other mammalian cells (Figure 5.6). Most human cells are loaded wall-to-wall with the components you may recognize from a high school biology text: organelles, mitochondria, a nucleus, etc. Removing any of those components will result in cell death. On the other hand, an RBC is filled with the oxygen-carrying protein, hemoglobin. While removing hemoglobin would prevent normal RBC function, it would not immediately kill the cell. Your body would continue to receive oxygen as bone marrow produces about 2 million new RBCs/second.

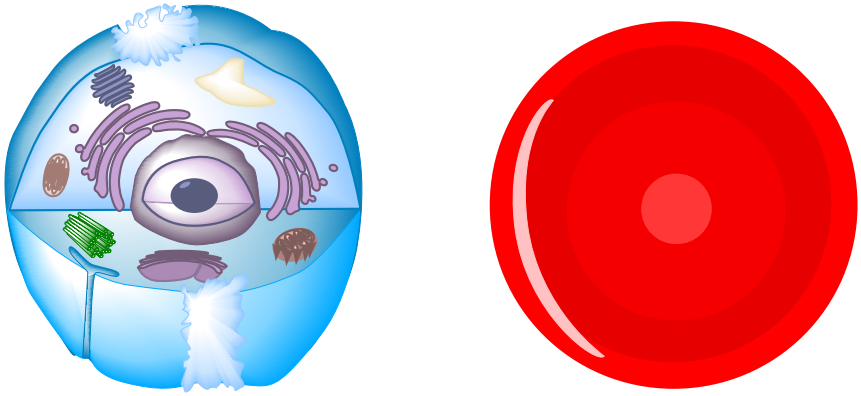

Figure 5.6. Advantages of RBCs over other cell types for drug loading. Typical human cells contain many critical components. Removing these components or altering the inside of a cell often results in cell death. On the other hand, RBCs are mainly empty inside except for the oxygen-carrying protein hemoglobin. Hemoglobin can be removed and other components added without destroying the cell.

To entrap the antenna-B12-drug design (Figure 5.1), we used osmotic gradients to open and reseal temporarily pores in the RBC membrane. Osmotic gradients are the difference in particle concentration between two sides of a semipermeable membrane, such as the inside and outside of an RBC.

As shown in the center panel of Figure 5.7, RBCs like to be in osmotic equilibrium, where the salt concentration is the same on both sides of the membrane (isotonic). If the concentration gradient is higher or lower on one side, the membrane will exchange water with its environment to offset that difference. For example, if the salt concentration is lower on the outside, water rushes in through the membrane to try and lower the salt concentration on the inside (hypotonic conditions). Because RBC membranes are flexible, water influx causes them to swell. If the salt concentration is higher on the outside of the membrane, water rushes out and the RBCs shrink (hypertonic conditions). But why do pores form? RBCs are often described as fluid-filled sacks held together with an elastic band. Pores form when the cell swells and reaches a maximum volume. If the cell swells too much, the membrane (aka the elastic band) will eventually break and kill the cell.

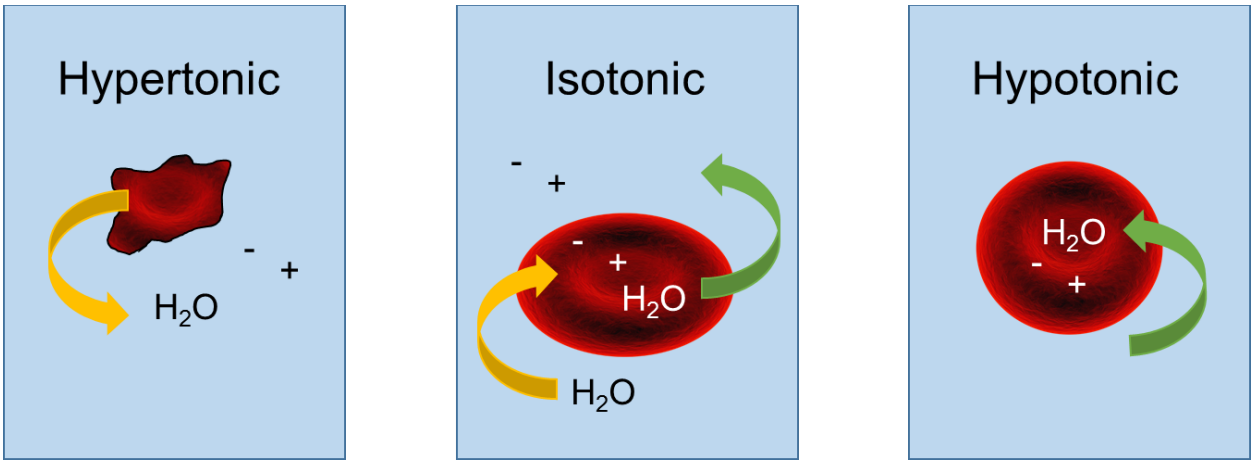

Figure 5.7. Osmotic gradients alter cell shapes. RBCs morph their shape when exposed to different salt environments. Hypertonic conditions (high salt levels) cause cells to shrink, while hypotonic conditions (low salt levels) cause cells to swell. Water either enters or leaves in an effort to maintain isotonic conditions (osmotic equilibrium).

Our approach to loading cells is to lower the outside salt environment so that the RBCs swell like a balloon. Instead of breaking, the ballooned cell forms pores through which our material rushes in while hemoglobin rushes out. We then increase the salt concentration again so the cells shrink back to their normal shape. Shrinking the cells effectively reseals the pores and encapsulates the drug scaffold (Figure 5.8).

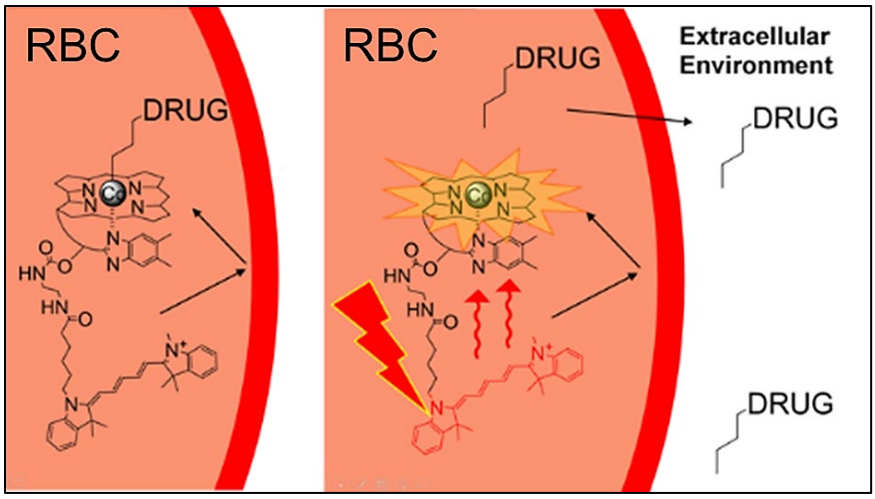

Figure 5.8. Encapsulating drugs into RBC interiors. To load RBCs, we place the cells in low salt conditions so that they swell and form pores through which antenna-B12-drugs enter. We then reseal the cells by increasing the environmental salt concentration, trapping the molecules. Finally, we release the drug by illuminating the cells with red light.

The cell interior provides exquisite two-way protection unavailable with membrane loading. It prevents other biological surfaces from pulling off the B12-drug while also protecting the body from any harmful effects of exposed therapeutics. While we continued our investigation of the anti-inflammatories methotrexate and dexamethasone, the RBC protective shield gave us the confidence to also explore cell-damaging chemotherapeutics, such as doxorubicin and paclitaxel. The debilitating side effects caused by chemotherapeutics are widely known. Using non-invasive light treatment to target cancer drugs directly to tumor sites has the potential to increase the effectiveness of an administered dose and greatly improve the quality of life for patients.

5.6. Generation 3: Re-design with Rodent RBCs and Pre-Clinical Studies

We were very excited to demonstrate that light-controlled release was possible from both the outside and inside of human RBCs. However, we quickly realized the limitations we would face when taking our technology to the next level. Human RBCs are the most relevant to *human* patients, but rigorous pre-clinical animal studies would be necessary before clinical trials could be considered. The next few months would exemplify the struggles faced while translating science from bench to clinic, the reality of unfruitful leads, and how challenges lead to more complete understanding and ultimate success.

As a chemistry lab, we lacked experience with the details of animal work. Our collaborators played key roles in the success of my project and it could not have been completed without them. We worked closely with the UNC Institutional Animal Care and Use Committee (IACUC) to design and receive approval for an animal protocol. Every experiment conducted with an animal must be approved by this committee, made up of at least one executive officer, a scientist, a non-scientist, a veterinarian, and a community member not affiliated with the institution. They rigorously review your proposed experiments to ensure the humane treatment of every animal in the institution. As we found out, this process often takes 1-2 months to complete.

Once the protocol was approved, we worked closely with both an arthritis clinician and a bioengineer who studies cancer. One of these collaborators recommended that we adapt our human RBC loading procedure to rat cells. Rats are larger and easier to work with than mice and our collaborator used them more often in his lab. No problem, I had spent the past year perfecting my RBC loading technique and certainly rat RBCs can't be that different from human RBCs, right? Wrong! For 6 months I struggled through countless trials and errors in my effort to load rat RBCs, only to watch them fall apart into a rubble pile of broken cells at the bottom of a tube every time. Finally, out of frustration, I decided to look at the rubble on a microscope to see what a pile of broken cells looks like up close. I was shocked! Huge crystals littered the slide, intermixed with seemingly untouched RBCs (Figure 5.9). I was expecting cell debris – not perfectly formed crystal structures!

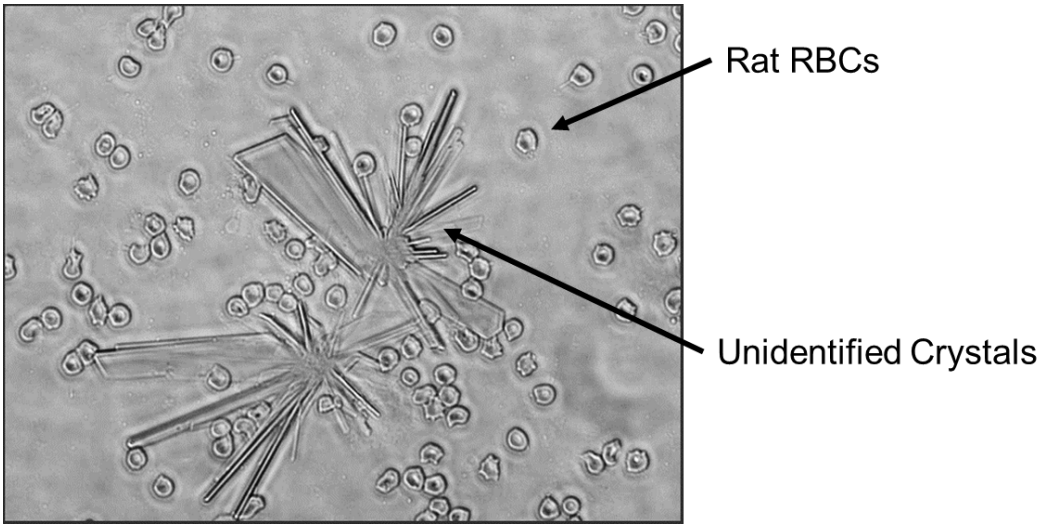

Figure 5.9. Unidentified rat RBC crystals. After months of failed loading attempts, I discovered that crystals had formed in the rat blood samples.

I eventually found a study from the 1980s in which a lab observed that rat hemoglobin, unlike any other hemoglobin in the animal kingdom, crystalizes when there is too much in solution. This limit was reached when we released hemoglobin through the membrane pores during loading. Through this process, we learned a vital lesson when working with RBCs from different species: Even though a human system and non-human system may look and behave very similarly, seemingly unimportant or unknown molecular differences may play the most important role in the success of biological inquiry.

After ensuring that mouse hemoglobin does not crystalize, we focused our attention on this alternative model. Once again, we immediately noticed differences between human and mouse RBC loading. Mouse RBCs are more fragile than human RBCs so we altered the loading procedure to ensure that the swelling and resealing were less harsh on the cells. At least this problem was workable! Even with a milder procedure, we do not expect mouse RBCs to last as long as their human counterparts in circulation. Like many pre-clinical evaluations, mouse experiments are the most relevant but are still not perfectly translatable to humans.

After the experiments were completed, we worked closely with the core facilities to process and help interpret our results. Core facilities are departments run by the institution that provide services and expertise in certain procedures. For instance, we wanted to ask the question: if we use light to deliver a drug to a certain spot on a mouse ear, can the architecture of the blood vessel tell us if the released drug is effective? To answer this question, we needed to prepare tissue slides, a procedure for which we did not have the equipment nor the expertise. We delivered the samples to the histology core, where their experts processed the sample, provided slides, and helped interpret the results. A majority of the figures in my final graduate school paper are a result of the teamwork between myself, my lab, and the multiple core facilities on campus.

5.7. My Results

All three generations of drug loaded RBCs provided us with a greater understanding of our system and prompted new ideas for improvement. The first generation, membrane decoration, demonstrated that we could attach our system to an RBC carrier and release drugs with long wavelength light antennas. As shown in Figure 5.10, we monitored drug release from B12-drugs modified with various fluorophore antennas Cy5 (646 nm), AlexaFluor700 (700 nm), Cy7 (747 nm), and DyLight800 (784 nm). We demonstrated that brightly colored LED lights only release the drug when the system is illuminated with the color that most closely corresponds to that of the antenna.

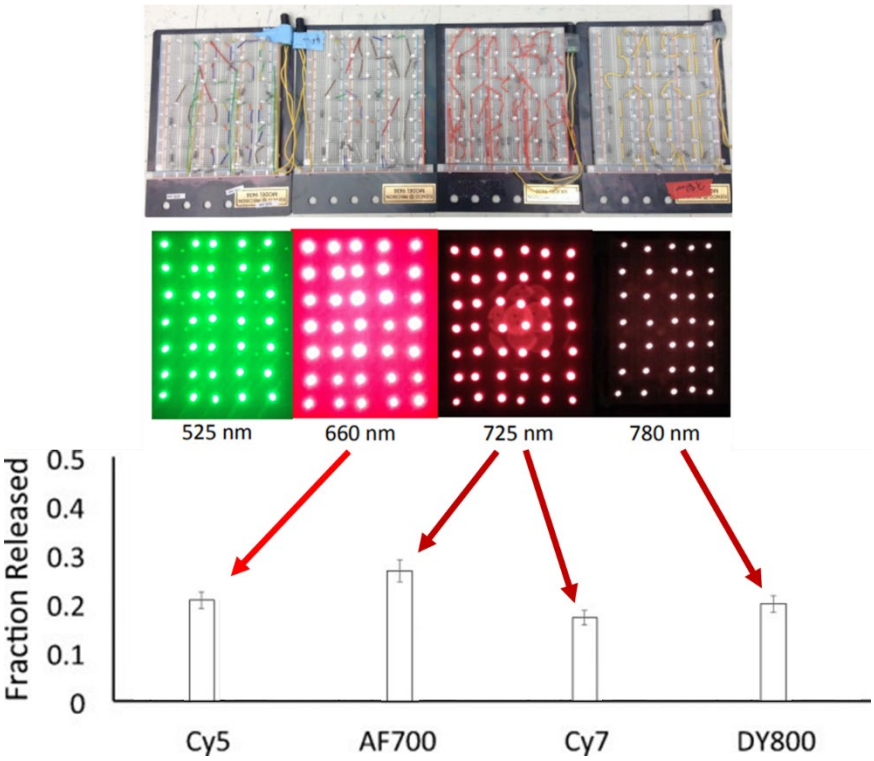

Figure 5.10. Release of a chemical group with long wavelength antennas. We attached a fluorescent molecule (representing a drug) to the Co-C position with various light antennas on 5′-OH and monitored molecule release from Co-C. Top: LED lights used to illuminate the loaded RBCs. Cy5, AF700, Cy7, and DY800 are 4 different antennas. Arrows represent which LEDs activate each antenna. Bottom: About 0.3 or 30% of the molecules loaded were released from each system.

Internally loaded RBCs provide more space and protection from the environment compared with membrane loaded RBCs, which prompted us to extend our therapeutic toolbox to include chemotherapeutics. To demonstrate that drugs released from the interior were effective, we mixed loaded RBCs with human cells taken from cervical tissue (HeLa cells) and observed effects of dark and light conditions on HeLa characteristics. Figure 5.11 represents one of these experiments using microtubules as indicative markers. Microtubules are normal cell structures needed for cell support and the released drug causes them to collapse. In the dark, microtubules spread out like a fan. Only when we illuminated the cells with the appropriate light did we see microtubules that resembled crumbled paper, confirming both light-induced release and the effectiveness of the released drug.

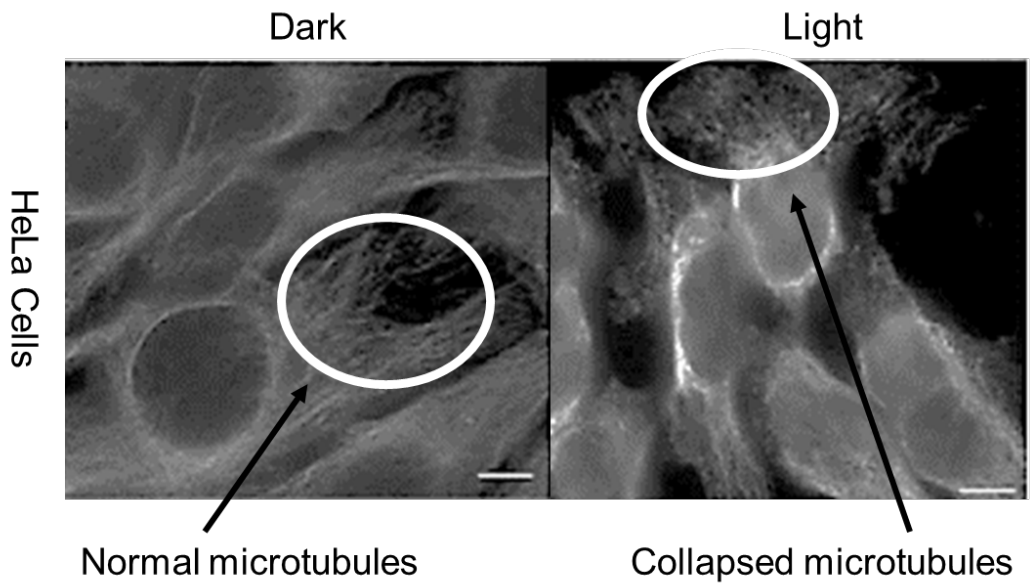

Figure 5.11. Effect of drugs released from RBCs after antenna-B12-drug encapsulation. Left: We demonstrated that loaded RBCs in the dark have no effect on the microtubules of human cells. Right: When the cells were illuminated, we observed microtubule collapse, a known effect of the released drug. These results suggest that illumination releases the drug and that the released drug is effective.

The third generation of drug-loaded RBCs was the most challenging to design and test. The need to switch from human to small animal RBCs forced us to confront molecular differences in seemingly identical RBCs and provided us with a broader understanding of RBC biology. I redesigned the loading procedure so that it was gentler and applicable to more fragile rodent cells. Using the vascular damaging drug, docetaxel, and a red Cy5 antenna, I confirmed area-specific release at a blood vessel in a mouse ear. Furthermore, I demonstrated that blood vessel architecture in a tumor could be compromised by site-specific release (Figure 5.12).

The first experiment was performed by observing the treated area with a microscope. You can clearly see bright red Cy5 fluorescence overtaking the screen around 1 hour, pointing towards blood vessel damage. In the second experiment, a tumor was imaged using ultrasound instead of microscopy. Here, blood vessel damage is more difficult to see by eye, but quantitative analysis of blood vessel leakage demonstrated that the drug was indeed working. While we learned that we can release effective drugs with our light-activated system, we are still exploring whether vasculature damage is enough to shrink the tumor. It may be that an even more powerful drug or a combination of drugs is needed – we are still looking for the answers to these questions!

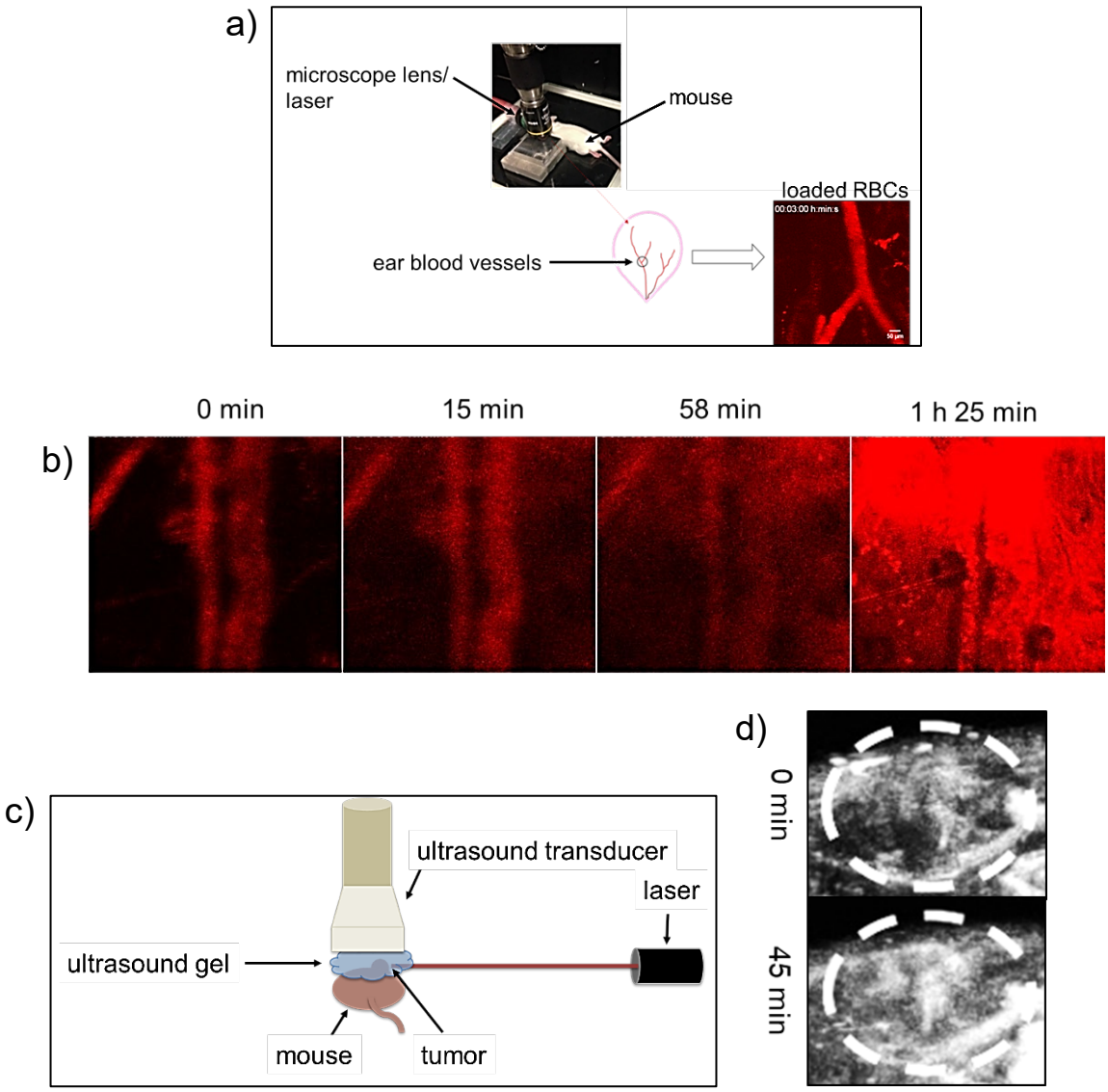

Figure 5.12. Preclinical studies with Cy5-B12-docetaxel-loaded RBCs. a) Intravital imaging setup. Loaded RBCs are fluorescent, so they can be seen flowing through blood vessels. b) Microscopy images of blood vessels in a mouse ear during light treatment. Fluorescence escaping from vessels demonstrates that blood vessels were compromised in a healthy mouse ear around 1 hour. c) Ultrasound imaging setup. d) Ultrasound images of a mouse tumor. In this case, light treatment was completed before imaging. Images confirm the microscopy results in that enhanced contrast agent (shown as white in the images, a compound injected to stain the vasculature) entered the tumor from vessels after treatment.

5.8. Conclusion

My work highlights the incredible teamwork and collaborations necessary to take an idea from the bench to preclinical studies. Often, multiple generations of an idea are necessary to arrive at a promising solution. I believe persistence and inquiry will teach us the most about the world and ultimately allow us to find ideal treatments for many horrible diseases. My graduate work resulted in the proof of concept for a new light-activated drug delivery system. The next steps will be to look more closely at disease models and determine whether this method has preventive or even curative properties more effective than our current drugs. I hope that the work in my dissertation will inspire both my lab's research and the work of other scientists and will one day be useful in clinical therapy.