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Hyperthermia as a Cancer Treatment- From Theory to Practice

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Hyperthermia as a Cancer Treatment- From theory to practice

A Thesis Presented

By

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1. Abstract

Using iron super-paramagnetic and ferromagnetic nanoparticles composed of Fe_3O_4 molecules, scientists analyze the effectiveness and practicality of this new treatment theory, hyperthermia. The problems of magnetic particle density, isothermal barriers/cellular cooling thresholds, and nanoparticle specific targeting are addressed in this review.

Iron magnetic nanoparticles were chosen due to their relatively low biological reactivities and lack of subsequent cellular toxicity. However, there are significant heating problems associated with these magnetic nanoparticles due to their relative size and short thermal time constants or thermal half-lives. Effectively, these aforementioned issues create a phenomenon where cancerous cells, surrounded by unheated healthy tissue, exhibit properties similar to those of an isothermal barrier. As a result, target cells experience limited gross heating, which is localized to the area directly surrounding the active magnetic nanoparticle within the cytoplasm. The effects of isothermal barriers and HSP up regulation on particle-based hypothermia are profound and prevent therapeutic temperatures from being achieved in single cell heating limiting the applications for Fe_3O_4 magnetic nanoparticle hyperthermia applications.

It has been shown that reaching a certain magnetic nanoparticle density within the cell can result in a larger heating capacity, though this effect is also dependent on the particle dispersion pattern within cytoplasm. It has yet to be concluded whether ferromagnetic particles or super-paramagnetic particles are superior or more practical for hyperthermic treatments as they each have distinct benefits, and further study is needed.

Finally, the popular targeting mechanism associated with magnetic nanoparticle research, monoclonal antibodies, require that they have an organic coating (such as starch) as a means of both providing an organic binding point and as camouflage for avoiding host filtration pathways. Forgoing this organic coating could lead to increased particle density within the cell and the adoption of a more specific targeting mechanism such as virus like particles (VLPs) altered to target HSP's could lead to an increase in

yield. Furthermore the up regulation of HSPs in response to therapeutic temperature is problematic for the therapies practically.

2. Introduction

Hyperthermia as a cancer treatment is a new and emerging field that uses heat as a means of inducing necrosis and apoptosis within a cancerous cell. While the idea of using heat as a means of combating cancer is relatively established dating back to the 1950's, new technologies now make the treatment practical, shifting it from the realm of theory to that of possibility. In this review I will be focusing on the use of Fe_2O_3 and Fe_3O_4 particles, the industry standards, as a means of inducing heat within a target cell, the various targeting mechanisms in development, the theoretical advantages and disadvantages of this therapy, and how these compare to more traditional cancer treatment methods.

a. What is hyperthermia as a cancer treatment?

Hyperthermia as a cancer treatment has taken a variety of different approaches utilizing a variety of different vectors to induce hyperthermia. Up to this point the term “hyperthermia as a cancer treatment” was used in an attempt to generalize the concept. The goal of particle based hyperthermic treatments is to preferentially subject cancerous cells to intense heat in order to induce necrosis (uncontrolled cell death), while limiting damage to surrounding tissues. There are a variety of vectors used to induce hyperthermia within cancer cells, including Fe_2O_3 (a magnetic particulate), Fe_3O_4 , gold nanoparticles, superparamagnetic iron oxide nanoparticles, and even localized deep tissue microwave. Each vector has its benefits and limitations, however for the sake of this review, we will focus on Fe_3O_4 as the primary hyperthermia vector, though Fe_2O_3 is also referenced.

A therapy is only as good as its targeting mechanism, in order for hyperthermia to become a reality new targeting mechanism must be developed that accurately control particle dispersion. Targeting via direct injection, monoclonal antibodies, and viral like particles (VLP's) are all potential delivery mechanisms for this therapy and the benefits and pitfalls of each is analyzed. The Fe_3O_4 magnetic nanoparticle responsible for particle based hyperthermic heating has historically required a starch coating as a binding platform for delivery mechanisms. Primarily this starch coating was used for monoclonal antibody targeting, though it had a secondary function of disguising the therapeutic drug from the host's immune system. The necessity of this starch coating as it pertain to VLP targeting and loading is analyzed through proposed experiments.

3. Historical Background

a. Traditional Cancer Treatment Methods

Before delving into the complexities of using hyperthermia as a cancer treatment, it is first important to establish a basic understanding of the cancer treatments most widely employed, those of chemotherapy, radiation, and surgery.

i. Chemotherapy Treatment

The history of chemotherapy as a treatment dates back to World War II, where, in the study of mustard gas-like chemicals, nitrogen mustard was discovered to act against cancer of the lymph nodes and paved the way for a series of drugs called alkylating agents (Berger, Aleri, Kalidas, Gadd, & Stump-Sutliff, 2014). These preliminary drugs worked by disrupting or destroying the DNA of cancerous cells and paved the way for other chemo drugs that target cancerous cells through a variety of means (Berger, Aleri, Kalidas, Gadd, & Stump-Sutliff, 2014).

However these early treatments, specifically those of chemotherapy and radiation are associated with the saying “the cure is worse than the disease” as they often caused profound issues associated with the treatment related toxicity. Many chemotherapy drugs target rapidly dividing cells within the body as a means of singling out cancer cells, which divide rapidly without any or most of the normal safe guards associated with healthy cellular mitosis (Muldoon , Soussain, Jahnke, Johanson , Siegal, & Smith, 2006). But cancerous cells are not the only cells within the body that rapidly divide, the cells of hair, mouth, nails beds and many others are likely to be affected by the chemotherapy drugs. These side effects can be painful both for the patient and effected families, especially when considering that cancer is generally asymptomatic for much of its progression.

ii. Radiation Treatment

Radiation as a treatment dates back to the early 1900’s where Professor Roentgen used radium to treat patients with cancer. However near the beginning of the twentieth century it was discovered that radiation could cause cancer as well as cure it. Eventually radiation resulted in the development of Conformal Radiation Therapy or (CRT), which uses CT (Computerized Axial Tomography) images to determine the location of cancerous masses in three dimensions.

Intensity modulated radiation therapy (IMRT) is similar to CRT but allows for the intensity of the beams to be adjusted controlling the amount of damage caused to the healthy surrounding tissue. (Berger, Aleri, Kalidas, Gadd, & Stump-Sutliff, 2014) . Again, this is an example of the dangers and limitations associated with traditional cancer treatments, radiation is limited to targeting cancerous tumors and has issues when considering cancers like leukemia and metastatic cancers (cancers that have spread throughout the body). Furthermore the use of radiation as a treatment damages surrounding tissue and can, in the case of head and neck squamous cell carcinoma, cause damage to salivary glands and profoundly impact the patients’ ability to swallow after treatment. (Langendijk, Doornaert, Verdinck-de Leeuw, Leemans, Aaronson, & Slotman, 2008)

iii. Surgery Treatment

Surgery is the final common, or widely used cancer treatment, which dates back centuries to Greek and Roman times. It was a Roman physician, Celsus who first wrote and observed, “After excision, even when the scar has formed, none the less the disease has returned.” referencing the prolific and virulent nature of cancer. While surgery as a means of cancer treatment is not new, surgery as a successful and semi-safe means of a cancer treatment dates to the 19th and 20th centuries. Surgery is often used in conjunction with other methods, but is privy to some of the same limitations associated with radiation, in that there needs to be a localized mass to be viable for surgery. In addition, surgery is an extremely intensive procedure often necessitating the removal of large amounts of the surrounding healthy tissue in order to ensure the complete removal of the tumorous mass. This extensive removal of healthy tissue can compromise the functionality of entire body systems or organs as surgeons are forced to remove healthy tissue to ensure total removal of cancerous cells, potentially weakening or kill the patient in the process (Birkmeyer, Sun, Wong, & Stukel, 2007). In addition any surgery has standard associated risks including those of infection, blood clots, and excessive bleeding.

4. Hyperthermia

How does hyperthermia fit into this discussion of cancer treatments? If surgery and radiation are able to target localized masses of cancer cells (tumors) what need do we have for another form of cancer treatment? Would our efforts be better spent perfecting and fine-tuning existing treatments as opposed to starting from scratch with an entirely new treatment?

First and foremost it’s important to consider some of the potential benefits associated with hyperthermia as a cancer treatment, benefits not necessarily found under the purview of the more traditional cancer treatments of surgery, chemotherapy, and radiation treatments.

Hyperthermia in conjunction with VLP targeting mechanisms has the ability to preferentially target cancer cells, without inducing necrosis within healthy cells that share some of the same features as their cancerous counterparts. Due to the physical nature of both targeting and heating, it is unlikely that healthy tissue will be directly targeted and even more unlikely that sufficient particle density will be achieved to activate the thermal processes. If any healthy tissue damage occurs it will likely be minimal as the body possesses an incredible capacity to dispel heat (Pearce, Giustini, Stigliano, & Hoopes, 2013) In essence particle based hyperthermic treatments have the ability to destroy a cell from the inside through induced heat and other mechanical factors that are not well understood. This specific heating would combine the targeted therapy of chemotherapy with the effectiveness of surgery and radiation while mitigating surrounding tissue damage. Furthermore with VLP delivery this therapy is able to target all cancerous cells within an organism at once, whether or not the tumor has metastasized becomes irrelevant as the whole body can be subjected to an electromagnetic field.

a. Hyperthermia in conjunction with other Cancer treatments

Thus far hyperthermia has been used primarily in conjunction with other cancer treatments. This is primarily due to the fact that subjecting cancerous masses to temperatures in excess of 113 degrees Fahrenheit can result in tumor shrinkage and the degradation of key proteins within the target cells' cytoplasm (van der Zee, 2002). The resulting reduction in tumor size can open the door to other treatment options that were previously not possible such as aggressive surgery.

Hyperthermia, similar to most cancer treatments, is generally considered to be most effective when used in conjunction with other cancer treatments such as radiation and chemotherapy. For the sake of this paper I will focus on the use of general chemotherapeutic drugs in conjunction with experimental hyperthermic treatments.

It has long been shown that the use of chemotherapeutic drugs on cancerous cells result in an up-regulation of heat shock proteins (HSP) within the cell (Ciocca & Calderwood, 2005). By first treating a

patient with classic chemotherapeutic drugs, cancerous cells would up-regulate their expression of HSPs, elevating previously high levels to even higher levels. Using the theoretical VLP loaded with Fe_3O_4 nanoparticles and tailored to target specific HSPs through alternations in VLP's viral entry proteins (e.g. HSP 70 and HSP 27 common in many cancers) the VLP complex would preferentially target the cancerous cell portraying these aforementioned HSPs on the cells external membrane (Multhoff & Hightower, 1996). This preferential targeting would result in a twofold protective mechanism that would limit surrounding tissue damage- particle density thresholds and preferential field targeting and activation.

Though all cells have a certain baseline level of HSP expression, it is more heavily expressed on the surface of cancerous membranes as a means of stabilizing the overly flexible membranes characteristic of cancerous cells. (Csoboz, et al., 2013) Any magnetic nanoparticles that found their way into healthy cells would likely lack the appropriate particle density to achieve therapeutic temperatures. In other words in order for therapeutic temperatures to be achieved a very high particle density must be achieved within the target cell, if a few of the particles mistakenly entered healthy cells there would not be enough of them to illicit damage (Pearce, Petyk, & Hoopes, 2015).

Furthermore, an electromagnetic field of varying strength can be localized to the specific area of interest within the subject. Take for example a hypothetical tumor in the abdomen subjected to this VLP Fe_3O_4 complex, while this complex is present in low quantities throughout the body, only the specific area in the abdomen is subjected to the electromagnetic field. While promising the idea of particle-based hypothermia is not without its flaws, the theory is still in its infancy and there are some serious issues that need to be addressed before practical and effective application becomes a reality.

b. Issues Associated with Hyperthermia

i. Super-Paramagnetic Heating vs. Ferromagnetic Heating.

Super-paramagnetic particles and ferromagnetic Fe_3O_4 particles each have their own unique physical properties and there seems to be no definitive results identifying the superior one for particle-

based hyperthermic application. The designation super-paramagnetic particles is given to magnetic particles whose hysteresis loop measured under static conditions has zero loop area or in other words the molecule does not have any magnetic flux under normal conditions, it is only under an induced field that the molecule magnetizes. Furthermore a super-paramagnetic field is characterized when a particle is small enough to only support one magnetic domain per core, and when the magnetic spins of particles are randomly oriented below their Curie point. (Pearce, Giustini, Stigliano, & Hoopes, 2013). Based on mathematical models and limited experimental evidence, (Pearce, Petyk, & Hoopes, 2015) analyzed the effectiveness of super-paramagnetic nanoparticles compared to ferromagnetic nanoparticles. Both particles were comprised of Fe_3O_4 crystals and the distinction between super-paramagnetic and ferromagnetic lies in the size of these crystal particles and their resulting properties (Pearce, Giustini, Stigliano, & Hoopes, 2013). The threshold between super-paramagnetic and ferromagnetic particles is still very much in debate with particle diameter cut-off ranging from 20nm to 100nm. But what are the implications of these distinctions?

It was previously proposed by Etheridge and Bischof that these super-paramagnetic particles possessed a higher specific absorption rate (SAR) in dispersed solution on a per mass basis. (Etheridge & Bischof, 2012) Ferromagnetic particles are particles whose diameter are larger than the previously mentioned cut-off diameter and experience the random magnetic spin orientation characteristic of super-paramagnetic particles above its Curie point. With regards to this material, Etheridge and Bischof found that in dispersed solutions these particles had a higher SAR on a per nanoparticle basis.

In the (Pearce, Giustini, Stigliano, & Hoopes, 2013) study they analyzed the specific properties of the Fe_3O_4 super-paramagnetic particles and its ferromagnetic counterpart. Throughout the study it was observed that in living cells magnetic nanoparticles tend to become segregated together within the cell through natural cellular processes. Within this study it was found that ferromagnetic nanoparticles were the more effective option when considering localized intracellular hyperthermia and this result was dependent on nanoparticle localization. However this conclusion is far from definitive (Etheridge &

Bischof, 2012) disagree with this conclusion and investigation into the distinction between these two particles is still very much under active research.

i. Targeting

Thus far hyperthermia via paramagnetic particles is still in clinical trials and is not widely available to the public. Most of the trials have used monoclonal antibodies and direct injection as a means of targeting specific membrane proteins of cancerous cells. However, there is a rather glaring issue associated with this this type of targeting mechanism. Localizing the magnetic nanoparticles to the cellular membrane periphery puts these particles in greater contact with not only the patients' blood and plasma, but also the surrounding tissue, increasing the time needed to enact the treatment and the danger to the surrounding tissue as the possibility of thermal eddies increases (Pearce, Giustini, Stigliano, & Hoopes, 2013).

1. Targeting via Monoclonal antibodies

Many researchers today are looking at monoclonal antibodies as a mean of targeting cancerous cells with their respective payloads. Targeting via monoclonal antibodies involves attaching an antibody (sensitive to a specific sequence) to the magnetic nanoparticle. This requires that the magnetic nanoparticle be cased in an organic compound to provide an anchoring means for the antibody. It's important to note that antibodies are highly specific and that they must be tailored to the cancer type of each individual. There are a variety of issues associated with this form of targeting that I will discuss through the paper.

It has been proposed by (Pearce, Giustini, Stigliano, & Hoopes, 2013) that magnetic particles in extremely close proximity to one another act as a solid. However experimental evidence produced results that differed from their predictive models, this may be because the bioshell required for antibody attachment keeps the particulates from interacting with one another in the manner they had predicted or that the bioshell itself acted as a heat sink. Furthermore monoclonal antibodies target the cellular

membrane without ever entering the cytoplasm of the target cell, effectively this puts the magnetic nanoparticle on the wrong side of the isothermal barrier.

2. Hyperthermia in relationship to membranes

As referenced earlier there are a variety of issues associated with the monoclonal antibody targeting mechanism. Monoclonal antibodies interact directly with the membrane of the cells periphery. Thus the magnetic nanoparticles molecules are localized to the cellular periphery as well. This interaction presents an additional issue when analyzing the chemical and physical nature of cancerous membranes and their response to hyperthermia.

First and foremost over the course of a cancerous cells existence they tend to progress towards a general shortening of their respective phospholipid acyl chains of the outer cellular membrane, this results in an increase in the general fluidity of all cellular membranes (Csoboz, et al., 2013). The cellular membrane of any given human cell serves as the primary means of heat stress detection through variations in membrane fluidity. Following the detection of thermo-stress cascade pathways are activated which up regulate expression of HSPs to stabilize the cellular function. (Csoboz, et al., 2013) Ultimately it has been found that it is the cellular membrane that acts as both detectors and effectors for cellular stress and in this specific situation dictate the adaptive hyperthermic cellular response. In essence, targeting cancer cells with monoclonal antibody Fe_3O_4 complexes limits the effectiveness of the magnetic nanoparticle by subjecting it the largest area of isothermal barriers and stimulates cellular process that make the cell resistant to heat. This limits the effectiveness of the magnetic nanoparticle while simultaneously increasing the resistance of the cancer cell to changes in temperature.

However, if it is possible to adapt a viral delivery mechanism, it would circumvent the membrane pathway limiting the initiation of thermal resistance pathways and the propagation of stress response molecules. In other words, by delivering the paramagnetic payload directly to the cytoplasm, it would limit the exposure to cellular membranes while focusing on the dissociation of key proteins within the

cytoplasm. Despite this relationship it is important to note that while cancerous human cells possess an incredible capacity for adaptation, there is a limit to the extent to which human cells can tolerate increased temperature.

Furthermore the increase in membrane fluidity presents a variety of issues for the hosts and benefits for the cancerous cells. This increase in membrane fluidity was suggested to be responsible for the extreme rates of proliferation and invasive potentials of the cancerous cells. (Csoboz, et al., 2013) In the specific case of cancerous cells with high metastatic potential, they exhibit high rates of enhanced membrane receptor mobility and reduced cholesterol/ phospholipid ratios. (Csoboz, et al., 2013)

5. Isothermal barrier and Particle Distribution and Density

Particle distribution and the size and distance between isothermal barriers were considered to be the two most important factors with regards to effective whole cell heating. (Pearce, Giustini, Stigliano, & Hoopes, 2013). It was found that larger particle density resulted in more effective heating of the target cell, though regardless of this effectiveness, single cell targets, or cancerous cell targets surrounded by isothermal barriers were unable to be heated to the necessary 6 degree Celsius increase in temperature. (Pearce, Giustini, Stigliano, & Hoopes, 2013) The isothermal barriers referenced are used as a mathematical explanation to illustrate the heat dispersing effects of single cells surrounded by unheated tissue and/ or fluids.

In the case of a single targeted cancer cell, the stimulated magnetic nanoparticle creates a localized area of heating within the cell while most of the cytoplasm undergoes no discernable change in temperature. In order to further corroborate the issue of isothermal barriers, (Pearce, Giustini, Stigliano, & Hoopes, 2013) calculated that an average cell entirely filled with the Fe_3O_4 magnetic nanoparticle and surrounded with unheated tissue (or isothermal barriers) would experience a temperature change of less than a degree Celsius. As a result there is a limited size capacity in which hypothermia via magnetic

nanoparticles composed of Fe_3O_4 could be effective. Interestingly the minimum effective size of a tumorous mass is around 2-3mm in diameter, this is because the heating of targeted cells surrounded by other targeted cells is relatively easy, resulting in the 6 degree change in temperature needed to induce cellular damage (Pearce, Giustini, Stigliano, & Hoopes, 2013). It's important to note however, that due to particle starch shielding only 30% of the total Fe_3O_4 particle complex was actually the active Fe_3O_4 particle.

a. Nanoparticle Preparation

Regardless of which size magnetic nanoparticle is employed (super-paramagnetic or ferromagnetic), the vast majority of studies emphasize the need for these particles to be coated within an organic, non-toxic material such as starch. The reason for this preparation is twofold; first, it is to provide a basis for the targeting apparatus such as monoclonal antibodies to bind to the surface of the target molecule and second, so that the target molecule is not filtered out of the blood via the kidneys. In the case of ferromagnetic particles it may be that the preparation is intended to keep the subunits distinct from one another for easier absorption. This is the case for ferromagnetic particles as they have a resting charge that would theoretically result in the clumping together prior to cellular absorption.

b. Nanoparticle Absorption

Absorption into the target cells is another important issue that relates to hyperthermia as a cancer treatment. It was found that smaller particles such as super- paramagnetic Fe_3O_4 particles, were absorbed less easily or rather were absorbed with a lesser degree of success because they were more easily washed away by blood (Pearce, Giustini, Stigliano, & Hoopes, 2013).

However, when Fe_3O_4 particles were injected directly into a mouse tumor, they saw less than a 20 percent retention rate of the magnetic nanoparticle within the cell. (Pearce, Petyk, & Hoopes, 2015).

Furthermore, when analyzing the volume of super-paramagnetic particles needed (BNF-Starch Nanoparticles) to heat a cell, it was found that only roughly 30% of the target molecule was composed of the active ingredient, Fe_3O_4 . The rest of the target molecule was composed of starch, when considering that it is Fe_3O_4 concentration that dictates the rate of hyperthermia; it is no surprise that such low increases in temperature were observed (Pearce, Giustini, Stigliano, & Hoopes, 2013).

As with any cancer treatment, the ability for cancerous cells to adapt to treatments is one of the main issues scientists face. Hyperthermia is no exception to this rule; through the increased expression of stressor molecules such as HSP's cells can achieve higher and higher rates of thermo-resistance. (Csoboz, et al., 2013) It is important to note however, that HSP is not the only molecule associated with that thermo-adaptation. Furthermore, it has been found that HSPs play a larger role in the regulation of cellular pathways as opposed to maintenance and protection of proteins. While HSPs have largely been characterized as the primary biologic obstacle for hyperthermic therapies, their high expression in cancerous cells could prove to be a benefit as it pertains to targeting.

6. Material and Methods

Hypothermia as a cancer treatment is an extremely new field with huge unknowns. It is a therapy very much in its infancy, and thus, there is still much to explore and many avenues of approach. Proof of concept experiments are required to test the ideas present in this review firstly an experiment to establish if Fe_3O_4 super-paramagnetic and ferromagnetic particles can be effectively loaded into a VLP, secondly an experiment to clarify discrepancies between ferromagnetic and super paramagnetic nanoparticles effectiveness and dispersion patterns and lastly an experiment to determine the necessity and function of the bioshell surrounding Fe_3O_4 magnetic nanoparticles.

a. VLP delivery mechanism

In order for particle based hyperthermic therapy to become viable a more specific and effective delivery mechanism is needed. As targeting the membranes of cancer cells via monoclonal antibodies is not a viable option (issues achieving particle density, targeting membranes illicit heat shock protein production, require a bioshell for Fe_3O_4 particles). Furthermore direct injection of magnetic nanoparticles into a tumor leads to inconsistent dispersion patterns with only a 20% uptake yield (Pearce, Petyk, & Hoopes, 2015). A possible solution for this issue would be to employ a VLP delivery mechanism as a means of effectively targeting cancerous cells in a specific and relatively controlled manner. This is possible due to the extreme vascular nature of tumors that aid in equal particle dispersion.

Virus-like particles are effectively virus shells comprised of either protein capsids, with or without membranes that lack the genetic information characteristic of classical viruses. While their use in medicine is much more controversial compared to monoclonal antibodies, they promise an effective new tool whose potential has only begun to be realized through its use in gene therapy, drug delivery and targeting mechanisms.

Using VLPs as a means of targeting for paramagnetic molecules would be effective in solving the primary issue associated with hyperthermic treatments thus far- particle density. Viruses come in a variety of shapes and sizes and as a result of this, their effective payload capacity also varies dramatically.

i. VLP payload size and selection

However there are benefits to using smaller viruses as opposed to larger ones, primarily that smaller viruses self-assemble in solution, facilitating both cost and ease of creation. In the case of *Macrobrachium rosenbergii* nodavirus (MrNV) VLP researchers were capable of encapsulating 2-3 DNA plasmids, with T=1 spherical symmetry and a diameter of 27 nm. While a VLP of this size would accommodate super-paramagnetic Fe_3O_4 particles, a larger VLP of closer to 20-50 nm would be needed to accommodate its ferromagnetic counter part. (Jariyapong, et al., 2014). Thus a large amount of Fe_2O_3 or Fe_3O_4 super-paramagnetic particles can be loaded into this example virus, which would in turn result in a

higher rate of particle density within target cells and a higher chance that sufficient particle density can be achieved within cancerous cell to induce cellular damage. When considering VLPs, size is a huge concern; super-paramagnetic particles are likely a better option as it pertains to VLP packing as a greater volume of them could be loaded into a single VLP. When considering ferromagnetic Fe_3O_4 particles it is likely only 1-2 particles could be fit into a large VLP of at least 100nm.

In order to establish that Fe_3O_4 particles can in fact be loaded into VLPs, a suitable VLP should be selected that can accommodate the size of an Fe_3O_4 nanoparticle within the ferromagnetic particle size range plus fluorescent marker (e.g. greater than 20 nm). HBsAg virus (Hepatitis B virus surface) can consistently be created in a range of 20-30nm. (Lopez, et al., 2017).

ii. VLP Practicality Experiment

In two independent experiments, a researcher should load un-constructed VLPs in a solution with encapsulated Fe_3O_4 plus fluorescent marker molecules. One experiment should use Fe_3O_4 particles in super-paramagnetic size range (under 20nm), in the other experiment Fe_3O_4 particle plus marker in the ferromagnetic size range (larger than 20nm) should be used. After VLP assembly solution should be purified and, using fluorescent markers, establish percent of VLPs within solution volume containing target molecule. Furthermore fluorescent intensity of singular VLPs can be analyzed as a means of determining per VLP molecular load.

b. Ferromagnetic vs. Super-Paramagnetic Fe_3O_4 and resulting dispersion pattern

The discrepancy between ferromagnetic and super-paramagnetic magnetic Fe_3O_4 particles as they pertain to effective heating needs to be definitively established. As stated earlier super-paramagnetic Fe_3O_4 molecules are easier to package, experience a per mass heating advantage, yet experienced lower cellular uptake values when direct injection into mouse tumors. Furthermore super-paramagnetic Fe_3O_4 molecules experience an advantage in heating at low field intensities, this is beneficial as the patients are less likely

to experience toxic eddy currents within tissues at lower field strengths. This means that the patient can be subjected to the alternating magnetic fields for longer periods of time.

Ferromagnetic molecules experience higher field strength advantages and ferromagnetic heating increases exponentially with field strength whereas super-paramagnetic heating experiences slower heating at higher field strengths. (Etheridge & Bischof, 2012)

Finally, dispersion patterns of magnetic nanoparticles within the target cell are another source of controversy. In the Etheridge and Bischof paper (Etheridge & Bischof, 2012) they state that dispersion as opposed to segregation have been shown to both positively and negatively affect heating rates. In contrast, Pearce *et.al.* (Pearce, Giustini, Stigliano, & Hoopes, 2013) state that segregation within the cell is vital for achieving therapeutic levels though they also state that having multiple high mass “clumps” within the cell would achieve the best result.

i. Ferromagnetic vs. Super-Paramagnetic Fe₃O₄ and resulting dispersion pattern experiment

Using the VLP synthesis outlined above, create two distinct VLP samples one with Fe₃O₄ super-paramagnetic load and another with Fe₃O₄ ferromagnetic load (both containing fluorescent marker). Inject super-paramagnetic nanoparticle into one group of mice and ferromagnetic nanoparticle into another group of mice. Mice should have tumors of similar size and cell composition as the type of cancerous cell determines rates of incorporation and dispersion. This should be done *in vivo* so that accurate VLP dispersion can be achieved.

Following VLP incorporation, tumors should be dissected and slides created and analyzed to determine not only the rates of cellular integration and dispersion patterns through fluorescent microscopy. Following cellular observation, samples should be placed in oil to mitigate the effects of the isothermal barriers. Samples should be subjected to electromagnetic field strength of 100kHz and 20kA/M. It is

possible that new samples without the fluorescent marker may need to be created, though this will affect load per VLP and subsequently dispersion patterns with no accurate way of measuring them other than looking at specific temperature changes within a specific cell.

c. Bioshell for magnetic nanoparticles

As a final experiment most literature reference particle based Fe_3O_4 molecules coated in organic compounds as a means of a bonding platform and to hide payloads from host immune system (Pearce, Petyk, & Hoopes, 2015) (Pearce, Petyk, & Hoopes, 2015). However, with the use of a VLP delivery mechanism there is no longer a need to sequester individual particles. In the case of Fe_3O_4 super-paramagnetic particle each individual particle has by definition a hysteresis loop of zero under controlled conditions. This means that each individual super-paramagnetic particle lack a magnetic charge under normal conditions. Thus there should be no issues with sequestering the particle into individual VLP's prior to activation.

Ferromagnetic particles do however have a small magnetic charge so loading them into specific VLPs without a coating buffer may present an issue. Another issue with regards to uncoated magnetic particles is that they lack a bonding platform for fluorescent molecules to adhere too. As a result, it will be very difficult to determine load per VLP and dispersion patterns of both ferromagnetic and super-paramagnetic molecules.

i. Bioshell for magnetic nanoparticles Experiment

After sample preparation purified VLPs will be subjected to an electromagnetic field and measure change in temperature. Should it prove possible to load both samples into VLPs and effectively administer them to mouse tumors, the tumors should than be dissected and slides prepared as in previous experiments. New mice samples should be prepared samples should be subjected to electromagnetic field strength of

100kHz and 20kA/M for 1min (using Helmholtz Coil or other set up) and heating intensity patterns and dispersion should be compared to previous experimental values to determine if there is a significant difference in heat localization and intensity. It was suggested that therapeutic temperatures were achieved after a minute of field activation at 100kHz using ferromagnetic Fe₃O₄ (Zou, et al., 2015).

7. Results

a. VLP practicality experiment results

It should be well within the realm of possibility to load encased Fe₃O₄ super-paramagnetic and ferromagnetic particles plus florescent marker into VLP's of appropriate size. Scientists should expect to see higher rates of both total VLP incorporation rates and load per VLP in the super-paramagnetic sample due to the relative sizes of the two molecules.

b. Ferromagnetic vs. Super-Paramagnetic Fe₃O₄ and resulting dispersion pattern experiment results

Based on my research I believe, but cannot definitively state, that ferromagnetic particles are better for achieving therapeutic heat levels than their super-paramagnetic counterparts. I am basing this rational on the Pearce, *et.al.* paper (Pearce, Giustini, Stigliano, & Hoopes, 2013), which suggest that the more ordered configuration of Fe₃O₄ molecules within the ferromagnetic structure leads to an increase in density of both the specific molecule and intra cellular molecular aggregates. Therefore I expect to see a shorter heating time and a greater gross temperature in the ferromagnetic sample.

c. Bioshell for magnetic nanoparticles experiment results

Lastly, the super-paramagnetic sample without the starch capsule should be able to be loaded into the VLP capsule without much difficulty. It has been hypothesized that one of the mechanisms in which super-paramagnetic molecules generate heat is through dipole-dipole interaction. Without the organic coating there will likely be an increase in this effect and perhaps even the creation of ferromagnetic particulates as super-paramagnetic particles reach threshold concentration.

8. Discussion and Conclusion

Hyperthermia as a cancer treatment is a new and exciting field whose potential has yet to be realized. Its extraordinary capacity to specifically target cancer cells in conjunction to VLPs capsules could revolutionize the way we see and treat cancers across the board. During the description of the possibility of utilizing VLPs as a delivery mechanism for these magnetic nanoparticles I neglected to mention a proposed targeting mechanism for the VLPs.

a. Theoretical targeting issues

The targeting mechanism behind any drug is often considered to be the most difficult part of designing any new drug/ therapy. Monoclonal antibodies have become the industry standard interacting preferentially with cellular markers on the surface of the cell but not physically entering the cell. This, as stated earlier, is an issue for a variety of reasons; first and foremost the outer cellular membrane is the primary sensor for the cell, specifically a heat sensor. (Csoboz, et al., 2013) After the detection of cellular stress, as such from hyperthermic therapy, the cell up-regulates its production of heat shock proteins (HSPs), which stabilize misfolded and unfolded peptide chains in response to cellular stress such as heavy metal exposure, fluctuations in temperature, and in response to toxins. (Ciocca & Calderwood, 2005)

b. Heat Shock Proteins (HSP) in relation to theoretical VLP targeting

In recent years HSPs have garnered interest within the scientific community for a variety of reasons including their role in chemotherapy resistance among cancer cells and as a means of preferentially targeting cancer cells. For many, the aim seems to be creating therapies and drugs such as Moxibustion that targets TRPV1 (Transient receptor Potential Vanilloid 1) and controls the subsequent expression of HSPs rendering a wide variety of chemo drugs more effective (Zou, et al., 2015).

However, I believe that this is ultimately futile as it is very likely we will eventually see a resistance to therapies of this nature. Using the up regulation of HSPs as a means of a targeting solution is a more effective long-term solution to the cancer issue.

Most human cancers are associated with an up-regulation of HSPs and their respective complexes (Csoboz, et al., 2013). This is likely due to the stressors experienced by rapid cellular replication resulting in inherent cellular instability. HSPs are up regulated in response to the hyper fluidity of cellular membranes characteristic of most cancers and to other cellular stressors. (Csoboz, et al., 2013) Specifically certain types of cancers exhibit specific up-regulations of certain HSPs, for example an up-regulation of HSP 27 is often characterized by gastric, liver, and prostate carcinomas and up-regulation of HSP 70 is characterized by breast, endometrial, uterine cervical, and bladder carcinomas (Ciocca & Calderwood, 2005).

Both of the afore mentioned HSPs are associated with chemo resistance via the blocking of apoptotic and necrotic signaling pathways. (Ciocca & Calderwood, 2005) It is however important to note that many cells within the body are constantly displaying a low level of a wide variety HSPs but in normal cells, this is a highly conserved and regulated pathway (Ciocca & Calderwood, 2005). So how does this pertain to a targeting mechanism if all cells within the body have a certain level of HSP expression?

c. Theoretical VLP Entry Mechanism

Membrane bound viruses are unprecedented in their ability to gain entry into host cells, generally, via membrane fusion. Membrane bound viruses employ a variety of mechanism to gain cellular entry but the most practical option within this context would be class one, fusion protein common in influenza hemagglutinin and retroviral Env (membrane gene) (White, Delos, Brecher, & Schornberg, 2008). Class one viral entry proteins are fairly simple in their design, self-contained and take advantage of a basic physical property- that merging membranes is energetically favorable (though there is a huge “activation energy barrier”).

To facilitate membrane merging, two separate class one viral fusion proteins (fig. 1) on the surface of the virus recognize and effectively lock on to specific membrane bound sequences. The receptor binding subunit, which just recognized the

sequence on the surface of the target cell, then dissociates from the six helix bundles as change conformations to create three hairpins that now span the distance between the viral and host membranes. These three hairpins (three hairpins for each complex so a total of 6 hairpins) then flex pulling the viral and host membranes together until the merge and the virus’s internal packaging enter the cell. (White, Delos, Brecher, & Schornberg, 2008)

Active research is looking into modifying the receptor binding subunit associated with class one viral fusion proteins (Stainslaw, Kalavathy, young, Hughes, & Chatterjee, 2011). If ultimately successful, the receptor subunit could be modified to recognize and respond to the HSP 27 and HSP 70 molecules found on the surface of stressed cells essentially targeting a marker present in most cancer cells regardless of type.

FIG. 1

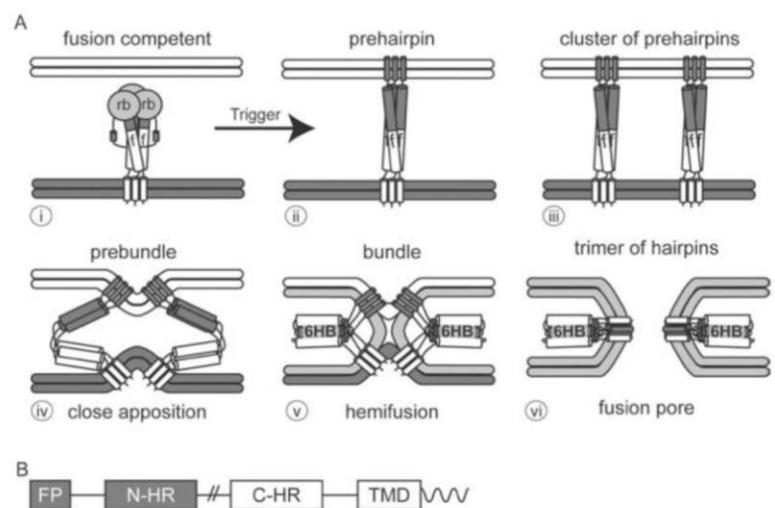


Figure1. Illustrates the mechanism of a class one viral entry protein. Taken from (White, Delos, Brecher, & Schornberg, 2008)

d. Outstanding Issues associated with Hyperthermic treatments.

As referenced earlier there are a variety of issues associated with hyperthermic treatments, specifically those of the Fe_3O_4 nanoparticles. The exact physical method of heating is still in debate with three possibilities Neel relaxation, Brownian motion, and perhaps particle-particle interaction in super paramagnetic nanoparticles. (Pearce, Giustini, Stigliano, & Hoopes, 2013) Regardless of the specific mechanism of action it was found that a certain particle density must be achieved to practically elicit a hyperthermic result. (Pearce, Giustini, Stigliano, & Hoopes, 2013). Particle distribution among the cancerous cells was of vital importance to the overall effectiveness of the treatment though they admitted that they did not take into account perfusion effects and that distribution of the nanoparticle via capillary networks within tumors would likely result in more effective and even particle distribution (Pearce, Giustini, Stigliano, & Hoopes, 2013). Ultimately the concept to take away from this paper is that to effectively illicit a hyperthermic response a certain particle density within a given area must be achieved. Taking into account the VLP targeting mechanism mentioned earlier it would be more likely that a larger volume of Fe_3O_4 could be delivered to the target cell.

The fact that hyperthermia via Fe_3O_4 magnetic nanoparticles can only be used on tumors with dimensions greater than 2 mm (Etheridge & Bischof, 2012) is a severe limiting factor on this treatment. In addition its inability to effectively kill all cancer cells within a tumor (those on the tumors periphery disperse heat faster than it is generated) limits its use as to supplemental therapy in association with other traditional cancer treatment methods.

Based on limited research data and extensive mathematical models Fe_3O_4 and Fe_2O_3 seem to be unable to achieve adequate heating to therapeutic levels in most scenarios within the clinical field strength of 100kHz 20kA/M. However in mathematical models used by Pearce *et.al.*, (Pearce, Giustini, Stigliano, & Hoopes, 2013) it was assumed that sequestered ferromagnetic particles would be sequestered at distance of 2nm and that this would in turn act as a solid. However, based on experimental data this may not be the

case, an experiment should be conducted where ferromagnetic shell degrades (perhaps pH sensitive shell) and subsequent heating is compared to coated ferromagnetic values. Ultimately, scientists are left with the question do we forgo Fe_3O_4 for larger more reactive but potentially more toxic magnetic/active particles or should we transition to less targeted versions of hyperthermia such as deep tissue microwave treatments.

i. Insulation of cellular membrane- theoretical solution to thermal- half-life of Fe_3O_4 magnetic nanoparticles.

The primary issue associated with Fe_3O_4 molecules is its thermal half-life and the corresponding isothermal barriers of unheated tissue/cell contact with target cells. There are however, avenues to explore that could potentially mitigate or solve these issues while maintaining the use of the relatively safe Fe_3O_4 molecule. During the direct injection of Fe_3O_4 magnetic nanoparticles via monoclonal antibody targeting solution, it was observed that unabsorbed particles acted as a thermal buffer mitigating the effects of the corresponding isothermal barrier. Thus far in all the papers I have researched, scientist have singularly used Fe_3O_4 as the sole active component of the therapy. But, as I mentioned earlier, there are theoretically treatments that could be employed that would prime and boost the effectiveness of the Fe_3O_4 ferromagnetic particulates.

The first theoretical conditioning would be increasing the selectivity of the VLP's Hsp70 and HSP27 targeting mechanism on the surface of the cancerous cell by treating cancerous cells to general chemo drugs to induce a stress response. Normally the VLP ferromagnetic complex would be introduced at this stage, however, there are further theoretically possible alterations that can be made to the VLP which would further increase the effectiveness of the hyperthermic treatment.

By incorporating a unique trans-membrane marker into the VLP membrane, it would provide an anchor for monoclonal antibodies attached to large insulating fatty molecules. It was found that fatty tissue experienced a 4X increase in heat retention compared to average tissue. (Pearce, Petyk, & Hoopes, 2015). Following VLP integration into cancer cell membrane ferromagnetic payload would be delivered to

cytoplasm and become sequestered by cellular processes. The unique trans membrane markers now on the cancer cells membrane would spread out quickly due to the extremely fluid nature of the cellular membrane. Monoclonal antibody and insulating complex would then interact with these markers insulating the cell and potentially mitigating the effects of heat dispersion/ isothermal barrier.

In conclusion hyperthermia as a cancer treatment rightfully remains in the realm of clinical trials and theory. Many of its associated issues need solutions before treatment becomes viable. However, if researches could make the treatment highly selective and achieve therapeutic temperatures in both cancerous single cells and localized cancerous masses the applications could extend well beyond the realm of cancer treatment. The versatile nature of this treatment, which combines physical and chemical therapies, has the capacity to redefine personalized medicine as we see it.

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Appendices

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