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# Controllable and Sequential Activation of Cancer Nanotherapy for Enhanced Synergistic Effect

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**Controllable and Sequential Activation of  
Cancer Nanotherapy for Enhanced Synergistic Effect**

An honors thesis presented to the  
Department of Biology,  
University at Albany, State University of New York,  
in partial fulfillment of the requirements  
for graduation with Honors in Biology  
and  
graduation from The Honors College

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Research Advisor: Prashanth Rangan, Ph.D.

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## Abstract

The treatment of numerous disease states has become increasingly more complex and challenging, even as we come out with new pharmacological and technological advancements. It is well known that cancer is not one disease, but many diseases that progress and present new challenges with each patient. With this we present a novel design which uses biorthogonal chemistry and magnetic nanoparticles (MNP's) to create a drug delivery system with the capability to deliver two drugs that are released at two different time points under the direction of a single trigger. This innovative combination of multiple advancements will allow for the treatment of normally resistant cancer phenotypes by rewiring the cell with one treatment, and then inducing apoptosis with the next, sequentially killing the targeted cell. Our system allows for a controllable, finely tuned delivery that can be targeted and imaged using MRI with our active MNP's. The system has been tested with release kinetics using two fluorescent probes, and then later *in vitro* to confirm efficient delivery of the payload. This simple, non-invasive treatment allows for a much easier and effective dual drug delivery system. It was shown using triple negative breast cancer cell lines to be more effective than current therapeutic treatments and enhance the efficiency of the drugs, when used synergistically.

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**Introduction:** Through the progression of many cancer types, biological changes occur throughout many parts of the genome that manipulate the cancer to resist numerous types of common therapeutic treatments. With this, many strategies have been theorized and attempted to fight this problem and propose new ways to deliver drugs<sup>1,2</sup>. It has been outlined by many sources including Woodcock et. al. that single dose treatments and many traditional drug therapies are becoming ineffective due to the complexity of new diseases, among these being cancer<sup>3</sup>. This brings about the need for potential therapies such as multidrug delivery, however, this also comes with many challenges such as an additional task for a patient, delivery of these drugs, and interactions between them. Furthermore, the side effects of chemotherapies are increased as well. Many delivery systems have been shown using methods with liposomes and micelles, for example, however there are many questions remaining on the efficiency and target efficacy of these methods to deliver multiple drugs in one platform. Due to this, we have implemented a system using magnetic nanoparticles (MNPs) with sequential release to deliver a multidrug cocktail into the cell. These MNPs allow for an image guided approach to therapy, along with their capability to load drugs, such that you can monitor the location and accumulation of drug in the patient<sup>4</sup>. MNPs also allow and extend the circulation time throughout the body, which gives a greater chance of uptake of the payload into the tumor<sup>5</sup>. Along with these MNPs, we have also employed the use of a special linker molecule<sup>6</sup>, which allows for a signal trigger molecule to release the drugs of multiple payloads at different times and provides a precise and highly controllable approach to the treatment of resistant disease types. While our initial study showed promising and successful results (N.M. Robertson, V.E.

LaMantia, M. V. Yigit, submitted), we herein explore additional chemotherapeutic agents to further expand the knowledge on this system.

### **Materials & Methods:**

Nanoparticle drug delivery is a very advantageous system to use and has picked up much popularity due to their inherent properties of solubility and specific number of binding sites. Concurrently, we employed the specialized linker system to allow for ‘click and release’ activation of one of the drugs immediately and another over a long period. Our MNP’s are an MRI-active amine terminated dextran coated iron oxide nanoparticle. These were previously used for noninvasive MRI imaging of lymph node metastasis in breast cancer patients, and prepared using the same methods as described within<sup>7</sup>. The linker system utilizes a bioorthogonal trans-cyclooctene (TCO) linker molecule which has been explored in many different areas, however has never been used for a practical delivery system<sup>6</sup>. This system utilizes an inverse electron demand Diels-Alder reaction (IEDDA) between TCO and tetrazine<sup>8,9</sup>. This allows us to functionalize nanoparticles with molecules of choice containing the TCO in one of two orientations, one in the axial position and the other in the equatorial position. The drug that will be released first will be determined depending on which drug is in which orientation. The drug placed in the axial position has a 156 fold faster release, which is believed to be due to steric hindrance and bond placement in the equatorial position.<sup>9</sup> The inherent IEDDA reaction allows for one molecule to be released immediately, followed by the other later. This becomes very useful for the treatment of resistant cancer types. This is because treatment in a sequential manner has found great promise. The activity of the first drug can rewire and reform the cancer



cell, after this the second drug can come in with a now more vulnerable cell and treat more effectively and kill the cell in a precise controlled manner. Whereas if these two drugs were added simultaneously, the treatment would not be effective<sup>1,10,11</sup>. The synthesis of these linkers and tetrazine are according to previous studies and can be found in the **supplemental section** below.

To study this method, we first used fluorescent dyes attached to the nanoparticles, rather than drugs. Using FAM ( $\lambda_{\text{ex}}$  484,  $\lambda_{\text{em}}$  517 nm), which was attached to the allylic position of the di-equatorial TCO (slow acting), and TAMRA ( $\lambda_{\text{ex}}$  550,  $\lambda_{\text{em}}$  576 nm), which was coupled to the di-axial TCO (fast acting) linker, we could see a distinct signal from each dye to determine release kinetics. Fluorescent studies were done using the Fluorolog3 spectrofluorometer.

Conjugation of the MNPs was performed as a mixture: 250  $\mu\text{L}$  of raw MNPs from stock, 250  $\mu\text{L}$  Sodium Citrate buffer (20 mM Sodium Citrate, 150 mM Sodium Chloride, pH 8.2), and 5  $\mu\text{L}$  of each dye in a 10 mM concentration. This mixture was left to conjugate in the dark for at least 24 hours. The mixture was then washed through a GE Healthcare PD-10 desalting column with Thermo Pack PBS buffer (100 mM PBS, 150 mM Sodium Chloride, pH 7.2). To show ratiometric loading, we conjugated 8 different sets of MNPs with various concentrations of each dye, from all TAMRA, to equal TAMRA-FAM, to all FAM. These were first imaged on the Bio-Rad Chemidoc with channels for both dyes and an overlaying channel to view both simultaneously. To further monitor the release of the dyes, we utilized High Performance Liquid Chromatography (HPLC) to show release after addition of tetrazine. These were conducted using a Phenomenex, Luna 5u C18(2) 100A column. Buffer A (100 mM TEAB pH 7.4); Buffer B (100% acetonitrile). The purification was achieved using an 18-minute gradient of 0-90% Buffer B.

Cell culture of MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's Modified Eagles Medium (DMEM). Media for the cells was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Ultrapure RNase-free water was used in all *in vitro* studies. All cells were cultured at 37 °C in a humidified incubator supplemented with 5% CO<sub>2</sub> per to suppliers' instructions.

For microscopy studies, ~1,000 cells were plated on MatTek glass bottom plates for 24 hours. These cells were then treated with ~534 nM (60 µg Fe/mL), 100 µL of the stock conjugated MNPs, and incubated for another 24 hours. The cells were washed and the medium was replaced with fresh DMEM 4 hours prior to confocal microscopy studies. The respective fluorescent channels were monitored throughout the study. We used the Zeiss LSM 710 Pascal laser confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) and analyzed using the Zeiss ZEN 2012 Confocal Microscopy Software for this study.

For cell viability, we used the previously well documented colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay<sup>4,12,13</sup>. On the first day of the study, using a 96-well format, ~100 cells were plated per well in 100 µL of DMEM and incubated for 24 hr. Day two, the cells were introduced to 5 µL of the stock MNP constructs (final concentration of ~508 nM MNPs, 57 µg Fe/mL) and incubated for an additional 24 hr. On day 3, the medium was removed and the cells were treated with 10 µM (1 µL of a 1 mM stock) of tetrazine in 100 µL of fresh DMEM and incubated for 32 hr. On the final day, the medium was removed, and cells were incubated with 100 µL of MTT solution (0.6 mg/mL in DMEM) per well for 4 hr at 37 °C. The MTT solution was then removed, and replaced with 100 µL of DMSO containing 4% aqueous ammonia per well to dissolve the purple formazan crystals. After 30

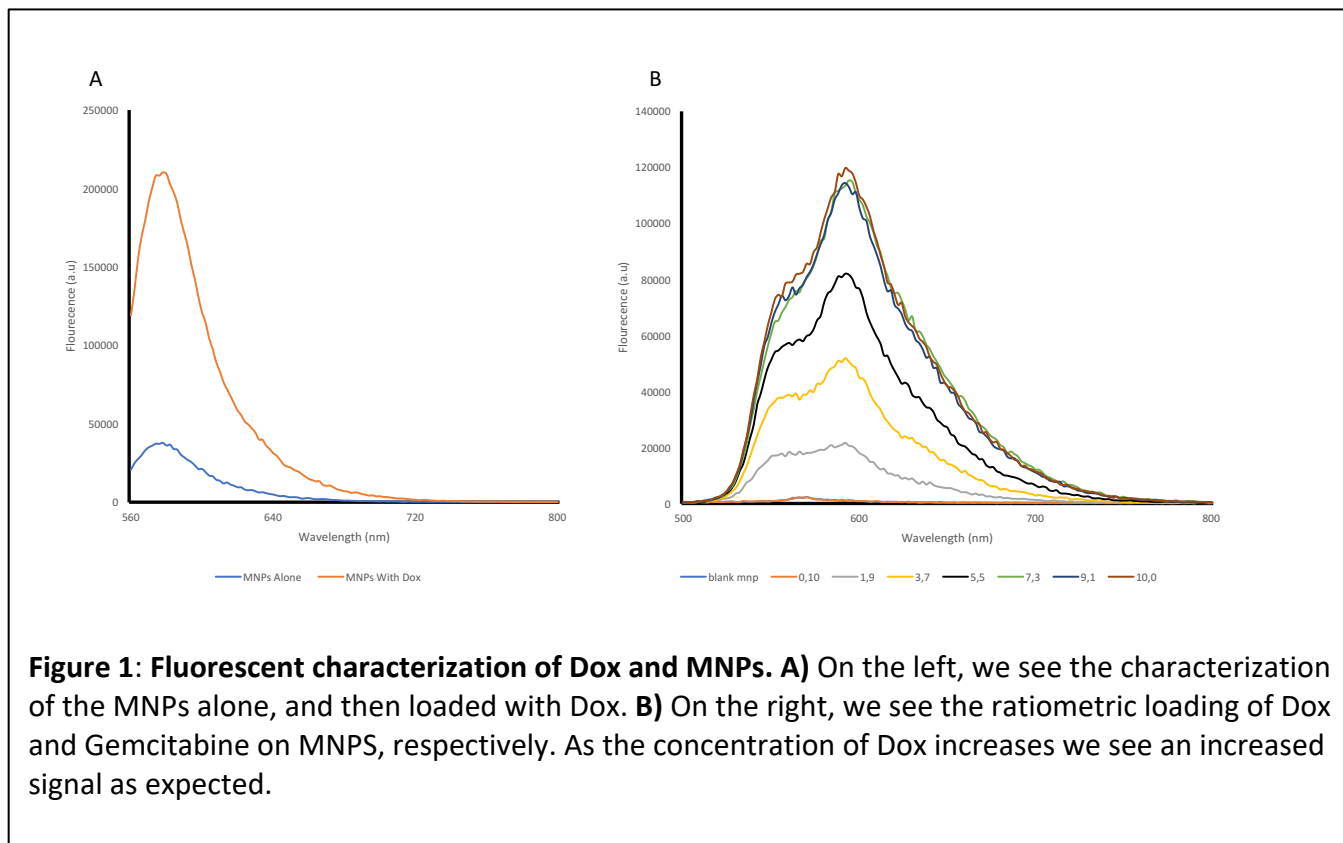
minutes, the absorbance of each well at 540 nm was recorded using a BioTek Synergy H1 microplate reader. Control studies were also done using wells only containing bare MNPs and bare MNPs with tetrazine to confirm the inactivity of the tetrazine on the cells viability. All assays were done in triplicates or quadruplets.

**Results:** To first show our MNPs conjugated with the dyes, as previously mentioned, we utilized the Chemi-doc imaging equipment. This clearly showed the correct ratiometric loading of our dyes in the various increasing and decreasing concentrations of our two dyes, and confirmed the lack of background signal from the MNPs. This was consistent with our previous studies (N.M. Robertson, V.E. LaMantia, M. V. Yigit, submitted). We then set out to show specific release using tetrazine, characterizing this through HPLC. Our MNP-TCO construct was incubated with tetrazine to characterize how the dyes would release from the nanoparticle and in what timeframe. After numerous trials, we established that the molecule on the fast linker would release almost instantaneously, and all the compound was released by the 30-minute timeframe. The molecule on the slow linker released starting at 48 hours after tetrazine addition and was completed by 96 hours (N.M. Robertson, V.E. LaMantia, M. V. Yigit, submitted) This gave us vital quantitative information about the exact release characteristics, and further incentive to work with pharmacological agents that would benefit from the specific release kinetics to maximize our systems potential to treat resistant cancer phenotypes.

Further studies were then done to look at this linker system in cell lines. Throughout this study, we used MDA-MB-231 triple negative breast cancer cell lines (TNBC). We initially wanted to look at cellular uptake and co-localization, to confirm that our MNPs were internalized, with confocal microscopy (N.M. Robertson, V.E. LaMantia, M. V. Yigit,

submitted). The overlay of both the FAM and TAMRA channels shows visually that both dyes are co-localized and internalized into the cells in an equal and partnered fashion in the same cell.

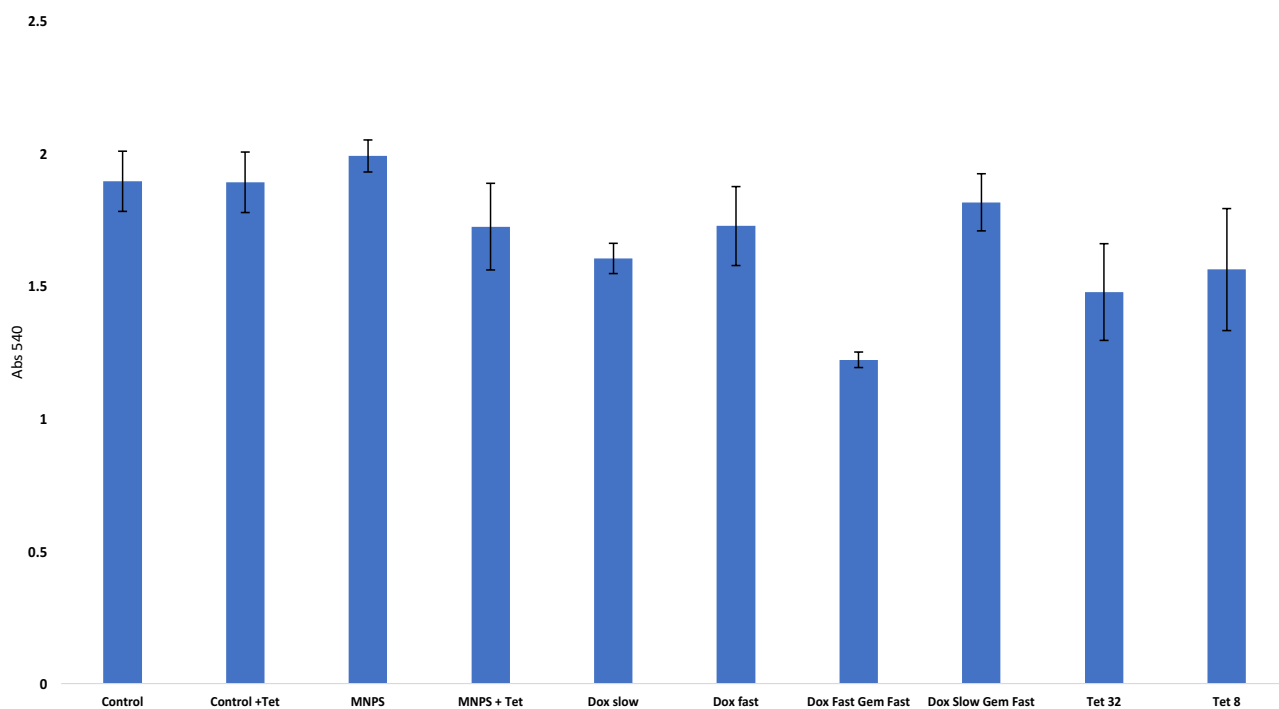
We next looked to characterize different chemotherapeutic drugs with a capability to attach to our linker molecule. Numerous studies have been done looking at different drug combinations and their respective efficacy<sup>2,9,10,14</sup>. However, there remains a need for a truly efficient and patient independent system. Our first study was done using Doxorubicin (dox) and Gemcitabine. Unfortunately, gemcitabine can't be view using UV/VIS technology as its absorbance peak is consistent with that of our MNPs and therefore can't be distinguished when attached. We did however characterize our MNPs with dox on the fluorolog-3 to ensure there was no background signal given off by the MNPs. The drug and MNP mix was conjugated as described above, and purified through the column. As seen in **Figure 1A**, there is a clear increase in fluorescence with the MNPs loaded with dox at the expected wavelength of about 580 nm.



We then further set out to show the ratiometric loading of a two-drug system in various concentrations to ensure equal loading onto the MNPs. While using dox and gemcitabine, we prepared the MNPs, washed them through a column as described above in ratiometric concentrations and then measured the fluorescence. **Figure 1B** depicts the loading of dox to gemcitabine, respectively, and shows that increasing the concentration of dox will show a higher fluorescent signal as expected. While we can see dox fluoresce here, unfortunately gemcitabine does not have fluorescent capabilities. However, the chemistry and techniques are the same here as they were for the loading of the dyes, so we are confident we can load both drugs equally and efficiently.

Once we proved we can link two payloads to our MNPs, and deliver, internalize, co-localize, and sequentially control the release of these payloads, we designed experiments to test the toxicity of our system on these cells. After characterization of the chemotherapeutics, and synthesis of the MNP-drug combination, we tested these methods on TNBC cells. TNBC breast cells have specifically shown a weakness when treated with Doxorubicin, a DNA damaging chemotherapeutic, when coupled with an additional agent in a time staggered manner<sup>1,10</sup>. Without this time staggered manner, the TNBC cells are resistant to conventional chemotherapeutics such as Dox<sup>1</sup>. Additionally, Gemcitabine, known as a pyrimidine antagonist, is a cytidine analog that has shown promise in treating a variety of cancers, most specifically non-small cell lung cancer, pancreatic cancer and bladder cancer. Its method of action in cells inhibits DNA replication by being converted into Gemcitabine 5'-diphosphate by active metabolites in the body. This compound inhibits ribonucleotide reductase, which is widely conserved across most species. Furthermore, the triphosphorylated derivative can become incorporated into the DNA, thus severely halting DNA synthesis<sup>15,16</sup>.

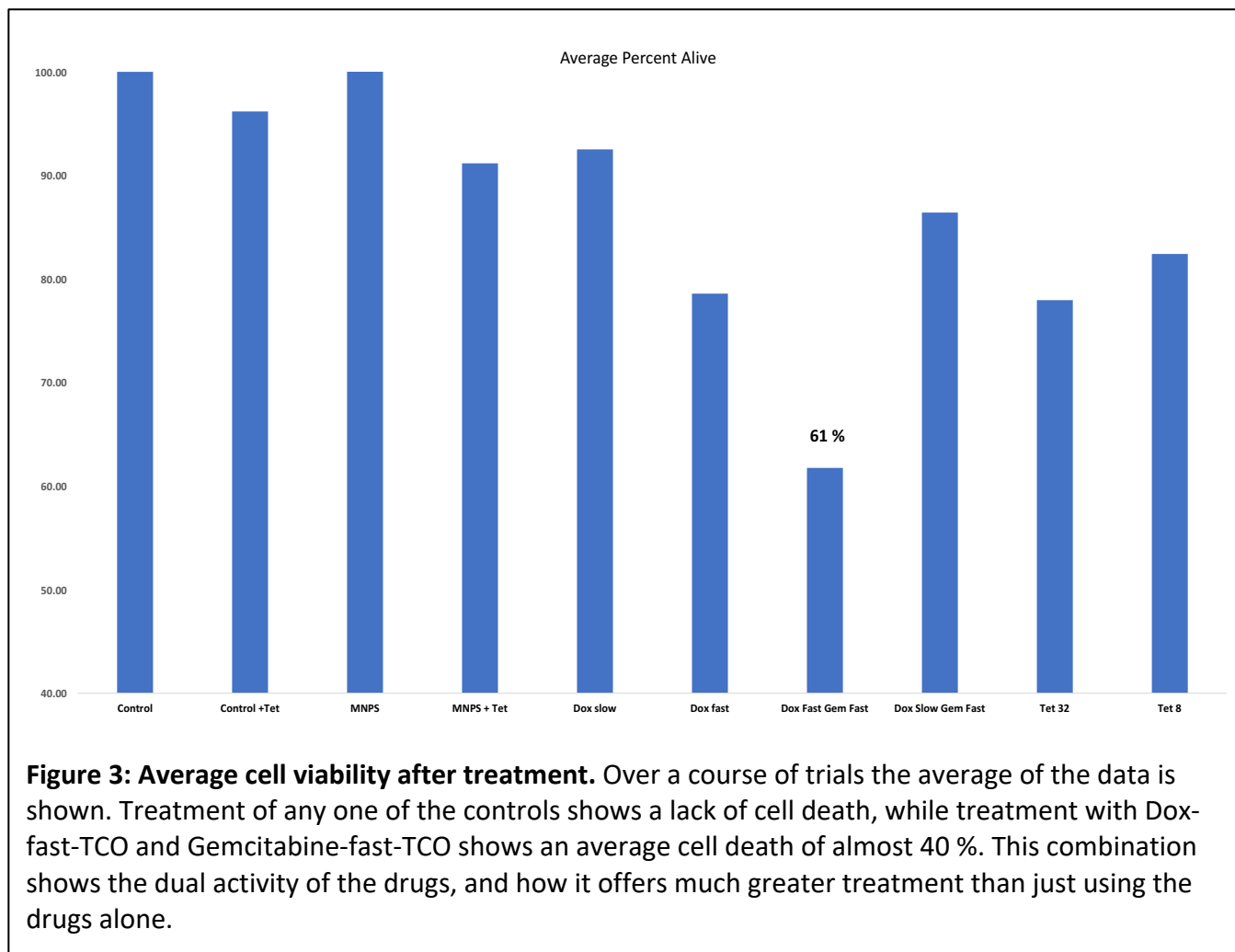
After displaying the click and release characteristics of the MNP-TCO-Drug constructs, we were then able to demonstrate this system in breast cancer cell lines to observe cell toxicity. Previously, studies were performed using PAC-1 and Doxorubicin as synergistic chemotherapeutic agents using the TCO linker system (N.M. Robertson, V.E. LaMantia, M. V. Yigit, submitted) and showed increased cell death in both BT-20 and MDA-MB-231 cell lines. Herein we look to expand on this knowledge and explore the effects of Gemcitabine and Doxorubicin. Control experiments were performed using just cells with no treatment, treatment with MNPs alone, treatment with tetrazine alone, and treatment with MNPs plus tetrazine. These studies showed limited cell toxicity as expected. We utilized different combinations of Dox and Gemcitabine, with different linkers, to explore which would have the best therapeutic effect on



**Figure 2: MTT Assay of MDA-MB-231 cells under treatment of MNPs.** This cell viability test shows the toxicity of various controls and treatments. The treatment of cells with MNPs or tetrazine (over both 32 and 8-hour time frames) shows little toxicity, while the combination of Dox-fast-TCO and Gemcitabine-fast-TCO shows high cell toxicity.

the MDA-MB-231 cells, with both the fast and slow TCO linkers. As seen in **Figure 2**, with Dox on the slow TCO linker and Gemcitabine on the fast TCO linker, cell death was minimal.

However, when utilizing both Dox and Gemcitabine on fast TCO linkers we saw enhanced cell death compared to the alternative combinations and the single drugs alone. This shows the enhanced functionality of the TCO linker system and how it can be utilized in several different ways. Over the course of several trials of this experiment we saw the same trends, which can be seen in **Figure 3**.



The average cell viability over the course of these trials, all conducted in triplicates or quadruplicates, was 61% for the Dox-fast and Gemcitabine-fast-TCO linkers. While our previous

literature (N.M. Robertson, V.E. LaMantia, M. V. Yigit, submitted) showed the best results utilizing the combination of slow and fast TCO linkers, our application using both the fast TCO linkers proved to be the best therapeutic treatment in this case.

**Conclusion:** Herein we have described and demonstrated the workings of a novel system to treat resistant forms of breast cancer using biorthogonal chemistry and magnetic nanoparticles. The combination of these two allows for loading of two drugs onto our MNPs and sequential or dual controlled release using a single tetrazine trigger. By using the TCO system and choosing which payload we put on the di-axial (fast) position or di-equatorial (slow) position, we can control the kinetics of the release. This allows for a controllable, systematic approach to treatment. We have outlined the loading of dyes on MNPs to characterize the TCO system, measured the release of these dyes through HPLC to show kinetics and imaged the fluorescence uptake of the MNPs *in vitro*. We then characterized two additional drug combinations that could be used to treat these phenotypically resistant cancer types, after initially showing the system in a different study (N.M. Robertson, V.E. LaMantia, M. V. Yigit, submitted). This system was shown to be effective to treat TNBC cell lines, which have been shown to be resistant to conventional treatments. This single trigger, dual release system has been shown to be effective for this treatment type, but ultimately holds promise in many other aspects of medicine because of the novel capability to load a variety of things onto the TCO linker system. This new approach offers a way to simultaneously or sequentially give treatment in a controlled and systematic way that was not before possible.



### Supplemental Information:

Nanoparticle Synthesis: The dextran coated superparamagnetic iron oxide nanoparticles were synthesized according to our previous reports (N.M. Robertson, V.E. LaMantia, M. V. Yigit, submitted) Briefly, 18 g of Dextran-T10 (Pharmacosmos, Holbaek, Denmark) was mixed with 60 mL of double-distilled water and stirred in a round bottom flask in an ice bath until dissolved completely. 1.3 g of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (Sigma Aldrich) was added into the clear dextran suspension while flushing Argon gas into the reaction mixture. 0.8 g of  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  (Sigma Aldrich) was dissolved in 5 mL of distilled water, which was previously flushed with Argon gas in order to avoid oxidation, and immediately added into the reaction mixture. 30 mL of concentrated cold  $\text{NH}_4\text{OH}$  (~28%) was added to the stirring homogenous mixture and the temperature was increased to 75-85 °C for an hour. The mixture was cooled to room temperature afterwards and concentrated to 40 mL using Amicon Ultra centrifugal units (MWCO 100 kDa; Millipore, Billerica, MA, USA). The dextran coating on the nanoparticles was cross-linked with the addition of 70 mL of concentrated NaOH (5 M) and 20 mL of concentrated epichlorohydrin (Sigma-Aldrich) and the mixture was stirred for 8 hours. Later, the nanoparticles were aminated by the addition of 120 mL of concentrated  $\text{NH}_4\text{OH}$  (~28%, Sigma-Aldrich) into the mixture and stirred for an additional 24 hr in the hood. The nanoparticle solution was purified using a dialysis bag (MWCO 14 kDa, SpectrumLabs, Dominguez, CA) against distilled water and finally suspended in 20 mM sodium citrate buffer (pH 8.0). The nanoparticle concentration was determined based on iron concentration, measured by UV-vis spectroscopy and adjusted to be ~10.0 mg Fe/mL.<sup>4</sup>

### ***Trans*-Cyclooctene (TCO) Synthesis and Chemical Conjugation.**

The NHS-TCO<sub>fast</sub>-NHS and NHS-TCO<sub>slow</sub>-NHS bioorthogonal TCO linkers utilized to immobilize the payloads on the MNP were synthesized according to the procedure described in the literature.<sup>17</sup>

*Synthesis of TAMRA-TCO<sub>fast</sub>-NHS:* TAMRA was synthesized based on the previously reported procedure.<sup>18</sup> The TAMRA (0.11 mmol) was combined with NHS-TCO<sub>fast</sub>-NHS (0.11 mmol) in DMF (2 mL), in the presence of triethylamine (100  $\mu$ L). The reaction mixture was stirred for 18 hr at room temperature under N<sub>2</sub>. DMF was removed under high vacuum and the crude product was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (3 mL). The title product (TAMRA-TCO<sub>fast</sub>-NHS) was obtained upon purification using preparatory SiO<sub>2</sub> TLC using a 9:1 mixture of CH<sub>2</sub>Cl<sub>2</sub> and MeOH, containing 2% triethylamine as a mobile phase. Yield = 50 mg (58 %).

*Synthesis of FAM-TCO<sub>slow</sub>-NHS:* The FAM was synthesized based on the previously reported procedure.<sup>19</sup> The FAM (0.17 mmol) was combined with the NHS-TCO<sub>slow</sub>-NHS (0.17 mmol) in DMF (4 mL), in the presence of triethylamine (200  $\mu$ L). The reaction mixture was stirred for 18 hr at room temperature under N<sub>2</sub>. DMF was removed under high vacuum and the crude product was redissolved in MeOH (3 mL). The title product (FAM-TCO<sub>slow</sub>-NHS) was obtained upon purification using preparatory SiO<sub>2</sub> TLC using a 9:1 mixture of CH<sub>2</sub>Cl<sub>2</sub> and MeOH as a mobile phase. Yield = 65 mg (54 %).

*Synthesis of DOX-TCO<sub>slow</sub>-NHS:* The compound doxorubicin (0.15 mmol) was combined with the NHS-TCO<sub>slow</sub>-NHS (0.15 mmol) in DMF (4 mL), in the presence of triethylamine (101  $\mu$ L). The reaction mixture was stirred for 18 hr at room temperature under N<sub>2</sub>. DMF was removed under high vacuum and the crude product was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (3 mL). The title

product (DOX-TCO<sub>slow</sub>-NHS) was obtained upon purification using preparatory SiO<sub>2</sub> TLC using a 9:1 mixture of CH<sub>2</sub>Cl<sub>2</sub> and MeOH as a mobile phase. Yield = 77 mg (60 %).

*Synthesis of Gemcitabine-TCO<sub>fast</sub>-NHS:* To a solution of Gemcitabine (50 mg, 0.19 mmol) in pyridine (5 mL) was added and TCO-bis NHS ester (80 mg, 0.19 mmol). The solution was stirred at room temperature for 18 hours. The reaction was quenched with H<sub>2</sub>O and concentrated to dryness under reduced pressure. The residue was dissolved in DCM and washed with 5% citric acid solution and brine. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and purified by flash silica gel column chromatography (5 % MeOH in DCM). The title product was obtained as a white solid. Yield = 59 mg (55%).

*Synthesis of tetrazine:* Isobutyronitrile (10 mmol) and zinc triflate (0.5 mmol) were combined with anhydrous hydrazine (1.6 mL) and stirred at 60 °C for 24 hr under N<sub>2</sub>. The reaction mixture was diluted with DMF (2 mL). An aqueous solution of NaNO<sub>2</sub> (3.5 g in 50 mL) was slowly added. Inside a thoroughly ventilated fume hood, an aqueous 2M solution of HCl was added slowly until reaching pH~3. (Caution! The last step generates highly toxic fumes, containing reactive nitrogen species.) The product was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x100 mL), dried with Na<sub>2</sub>SO<sub>4</sub> and concentrated. The title product was obtained by chromatography using 1% Et<sub>2</sub>O in pentane (1.2 g, 72%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ: 3.62 (sep, *J* = 6.8 Hz, 2H), 1.51 (d, *J* = 6.9 Hz, 12H). <sup>13</sup>C{<sup>1</sup>H} NMR (CDCl<sub>3</sub>, 100 MHz) δ: 173.69, 34.14, 21.22. HRMS (DART) *m/z*: calcd. for C<sub>8</sub>H<sub>15</sub>N<sub>4</sub> [M+1]<sup>+</sup> 167.1297; found 167.1306.

**Nanoparticle Conjugation:** The amine terminated iron oxide nanoparticles (MNPs) were functionalized with NHS modified molecular cargoes as follows. For the conjugation of the small molecule dyes, 250 μL of stock MNPs (7.2 mg Fe/mL, 64 μM) was added to 750 μL of citrate buffer (20 mM Na-Citrate, 150 mM NaCl, pH 8.2). 100 μL of this mixture was then

combined with another 50  $\mu\text{L}$  of citrate buffer and the TAMRA-TCO-NHS and FAM-TCO-NHS in a ratio out of 10  $\mu\text{L}$  (0:10, 1:9, 3:7, 5:5, 7:3, 9:1 and 10:0  $\mu\text{L}$  from 10 mM stocks) for a final volume of 160  $\mu\text{L}$  and MNP concentration of 1.125 mg Fe/mL (10  $\mu\text{M}$ ). These mixtures were incubated in the dark at room temperature for 24 hr and purified afterwards with Sephadex PD-10 columns against PBS buffer. The nanoparticle band in the column was collected whereas the free unconjugated dye band was discarded. For characterization of the dye conjugated MNPs [FAM-TCO<sub>slow</sub>-MNP-TCO<sub>fast</sub>-TAMRA, (MNP-F<sub>s</sub>T<sub>f</sub>)], the absorbance spectra were measured and standardized to the Abs<sub>360</sub> of the bare MNP solution. For the conjugation of the small molecule drugs, 250  $\mu\text{L}$  of stock MNPs (7.2 mg Fe/mL) was added to 250  $\mu\text{L}$  of citrate buffer. To this mixture, 50  $\mu\text{L}$  of the 10 mM stock aqueous solutions of each drug conjugate, DOX-TCO<sub>slow</sub>-NHS and PAC1-TCO<sub>fast</sub>-NHS, was added individually or in combination to obtain various MNP-TCO variations: [DOX-TCO<sub>slow</sub>-MNP-TCO<sub>fast</sub>-PAC1, (MNP-D<sub>s</sub>P<sub>f</sub>)], [MNP-TCO<sub>fast</sub>-DOX, (MNP-D<sub>f</sub>)] and [MNP-TCO<sub>fast</sub>-PAC1, (MNP-P<sub>f</sub>)] at a final volume of 600  $\mu\text{L}$ . An additional 50  $\mu\text{L}$  of citrate buffer was added to each mixture when necessary to obtain 600  $\mu\text{L}$ . After 24 hours of incubation in the dark at room temperature the conjugated MNPs were purified with Sephadex PD-10 columns against PBS buffer and the concentration was adjusted to be 1.2 mg Fe/mL (10.7  $\mu\text{M}$ ) using PBS buffer for *in vitro* studies.

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