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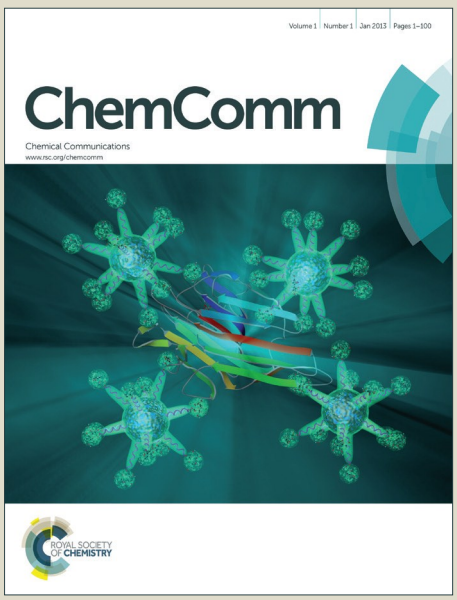

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ARTICLE

In situ activation of a doxorubicin prodrug using imaging-capable nanoparticles

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A general strategy for image-guided prodrug activation using fluorescently labeled magnetic iron oxide nanoparticles is described. It is based on a recently developed concept in bio-orthogonal inverse-electron demand Diels Alder chemistry termed 'click-to-release'. To illustrate a potential new biomedical application of the chemistry, the nanoparticles were modified with tetrazine, as well as near infrared fluorescent (NIRF) cy5.5 dye, while doxorubicin was converted into a prodrug. The nanoparticles taken up by the MDA-MB-231 breast cancer cells efficiently converted the prodrug of doxorubicin into the biologically active chemotherapeutic doxorubicin form.

Introduction

The inverse electron demand Diels-Alder (IEDDA) reaction between *trans*-cyclooctene (TCO) and tetrazine has been explored for a number of biomedical applications capitalizing on fast reaction kinetics, as well as bio-orthogonality of the chemistry to functional groups found in biological milieu.[1] The majority of the described constructs involve TCO-conjugated antibodies that deliver the bio-orthogonal reagent to antigen-specific cells or tissues.[2] Meanwhile, tetrazine is attached to therapeutic or imaging modalities, thereby facilitating targeted drug delivery, radio-imaging, or pathogen detection.

Recently IEDDA chemistry has also been explored on the surface of nanomaterials, such as magnetic nanoparticles (MNPs). These applications are particularly intriguing, as bio-orthogonality of TCO and tetrazine are complemented by the non-toxic dextran-coated MNPs that were clinically used for lymph node imaging.[3] Enhanced permeability and retention (EPR) effect and receptor-mediated endocytosis facilitate MNP delivery of therapeutic payloads to cancerous cells and tumor tissues.[4] In addition these MNPs are T2-weighted MRI contrast agents, capable of non-invasive imaging.[5] In recent reports, tetrazine-conjugated MNPs have been utilized for the detection of Gram-positive bacteria,[6] profiling of glioblastoma or erythrocyte-derived or microvesicles.[7] Similar constructs were also reported to be diagnostic tools for the detection of lung cancer.[8]

The current work describes a new biomedical application for tetrazine and NIRF-modified MNPs using recently reported 'click-to-release' chemistry, which is yet to be explored on the surface of nanomaterials.[9] We illustrate how this new development in IEDDA chemistry can be used for image-guided prodrug activation inside of cancer cells. This strategy has the potential for converting systemically administered prodrugs into image-guided targeted chemotherapeutics.

The conventional chemotherapy relies on the systemic injection of active drugs into the blood circulation en route to the cancerous tissues, where they will bind the intended target and exhibits their therapeutic functions. An ideal drug would concentrate only at its intended target, but the vast majority of the existing small molecule drugs distribute throughout the entire body. Because of a lack of specificity of chemotherapeutic agents for the targeted pathological site, large doses are typically required to achieve effective local concentrations.[10] These high doses chemotherapeutic drugs often result in non-specific toxicity and other adverse side effects.

Meanwhile, systemically administered non-toxic MNPs accumulate primarily in liver, spleen and lungs but also home in cancerous tissues due to EPR effect.[11] Uptake of tetrazine and NIRF-labeled MNPs by the cancer cells can be monitored *in vitro* by microscopy or *in vivo* using T2-weighted MRI. Once localization of MNPs inside the cancer cells is confirmed, they can act as molecular triggers capable of activating systemically administered chemotherapeutic prodrugs containing releasable TCO. Thus, the development of image-guided, activatable prodrug delivery approaches will minimize the side-effects related to drug's penetration of healthy cells and will allow precise control over prodrug activation in the target cancer cells.

The procedure described herein consists of two steps and involves two reagents: MNPs modified with tetrazine and NIRF cy5.5 dye, (**MNP-cy5.5-tet**) and a prodrug of doxorubicin, formed by modification of the latter with releasable TCO (**DOX-TCO**). Cytotoxicity of doxorubicin is significantly attenuated by the chemical modification, **Figure S1 and S2**.

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† Electronic Supplementary Information (ESI) available: Experimental details, synthesis and compound characterization, SI figures. See DOI: 10.1039/x0xx00000x

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MNP-cy5.5-tet acts as a molecular trigger capable of converting **DOX-TCO** into doxorubicin (**DOX**) inside of live cells.

The image-guided activation of **DOX-TCO** can be selectively achieved inside of cells upon systemic administration of **MNP-cy5.5-tet**. MNP accumulation can be monitored by fluorescence microscopy. Once, adequate accumulation is confirmed, **DOX-TCO** is systemically administered at the concentration that correlates to its IC_{20} value. The prodrug's therapeutic activity multiplexes when it reaches the cells containing **MNP-cy5.5-tet** that triggers the prodrug's conversion into doxorubicin which is considerably more cytotoxic than the **DOX-TCO**.

The underlying mechanism of prodrug activation is illustrated in **Figure 1**. The multivalency of MNPs allows loading of up to about 60 tetrazines per nanoparticle. After the bio-orthogonal IEDDA reaction ('click' step) the cycloaddition product spontaneously tautomerizes, thereby converting **DOX-TCO** into **DOX** inside the target cells ('release' step). Herein, we describe the synthesis of **MNP-cy5.5-tet** and **DOX-TCO** and illustrate how image-guided prodrug activation can be achieved in MDA-MB-231 human breast cancer cells.

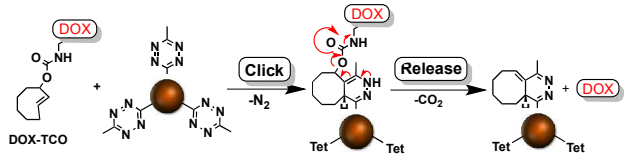

Figure 1. Illustration of how click-to-release chemistry is utilized on the surface of MNPs for activation of the **DOX-TCO** prodrug.

Results and Discussion

Synthesis of DOX-TCO and MNP-cy5.5-tet

The prodrug of doxorubicin, **DOX-TCO,** was synthesized as shown in **Figure 2**. The amine group of **DOX** was modified with TCO, using releasable carbamate linker.[9] The details of the synthesis are described in the supporting information. This structural modification substantially lowered potency of the drug. The cytotoxicity studies, shown in Figures **S1** and **S2**, revealed that that **DOX-TCO** is 5-times less cytotoxic than **DOX** to MDA-MB-231 cells. From the MTT assays, the IC_{50} values of **DOX** and **DOX-TCO** were determined to be 97 and 480 nM, respectively.

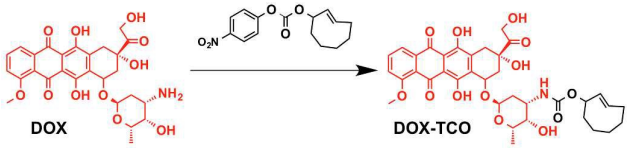

Figure 2. Synthesis of cleavable **DOX-TCO** prodrug from **DOX**.

Meanwhile, the nanoparticles capable of activating **DOX-TCO** were synthesized, as described in **Figure 3a**. The polydextran coated iron oxide nanoparticles, averaging 25 nm in diameter with about 180 amine termini on the surface were used. The amine groups were concomitantly coupled to Tet-NHS and cy5.5-NHS in PBS buffer (pH 7.4). After the 24 h coupling, the nanoparticles were purified by size exclusion chromatography. The MNPs with cy5.5 label show strong fluorescence emission at 710 nm which was absent with the unmodified MNP, **Figure 3b**. Ratiometric functionalization of MNPs with variable amounts of Tet-NHS and cy5.5-NHS is shown in **Figure 3c**. Difference in the visual appearance (**Figure 3c**) and spectrophotometric properties of the five different sets of MNP suggests that the amount of each functional moiety on the surface can be controlled by the ratiometric approach (**Figure 3d**). Modulation of the two functional groups allows to achieve the construct with optimal fluorescence for validating MNP cellular uptake and capability to trigger **DOX-TCO** activation for optimal therapeutic outcome. In the subsequent studies, such construct was deemed to be MNPs synthesized using 1:9 ratio of cy5.5-NHS to Tet-NHS.

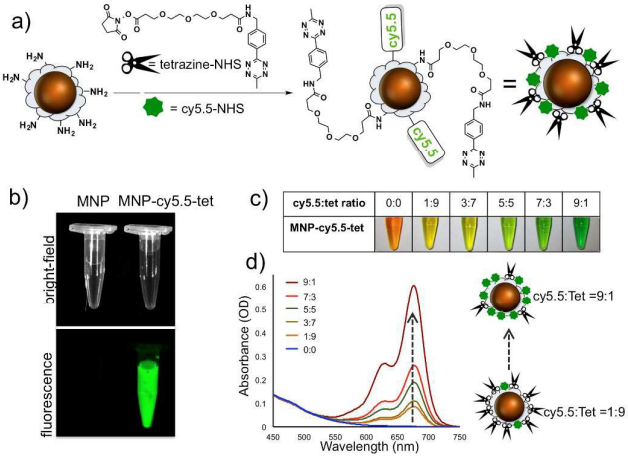

Figure 3. (a) Illustration of MNP functionalization with cy5.5-NHS and tetrazine-NHS to form **MNP-cy5.5-tet**. (b) The unmodified nanoparticles lack any fluorescence, but **MNP-cy5.5-tet** is fluorescent (exct: 675 nm, emm; 710 nm). The cy5.5 and tetrazine content on MNP surface can be controlled ratiometrically and monitored (c) visually and (d) spectroscopically.

Prodrug activation *in vitro* and in live cells

Prodrug activation was first tested *in vitro*. Aqueous solutions of **MNP-cy5.5-tet** (2 μM) were treated with 0.2 μM **DOX-TCO** for variable amounts of time. The solutions were separated from nanoparticles using amicon tubes. The filtrates were concentrated and analyzed by HPLC. The HPLC spectra of five different supernatant solutions, collected after different incubation times, are shown in **Figure 4**. The prodrug activation begins almost immediately after addition of **DOX-TCO** to **MNP-cy5.5-tet**. Complete conversion of **DOX-TCO** to **DOX** has been observed after 1 h of incubation.

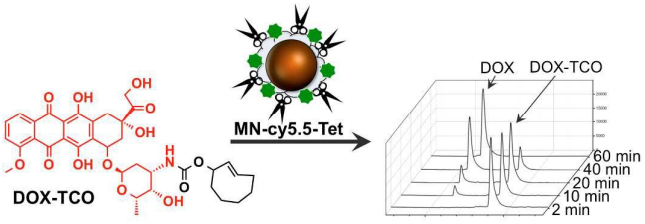

Figure 4. Activation of **DOX-TCO** prodrug using **MNP-cy5.5-tet**. The HPLC data showing the disappearance of the **DOX-TCO** peak and appearance of the **DOX** peak over time.

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The prodrug is efficiently taken up by the MDA-MB-231 cells (**Figure 5a**). The image-guided *in situ* prodrug activation was subsequently tested using the same cell line. Approximately, 1000 cells were grown in 25 mm MatTek glass bottom dishes and treated with **MNP-cy5.5-tet** (2 μM) for 24 h. The nanoparticle uptake was confirmed by fluorescence microscopy measurements, shown in **Figure 5b**. Subsequently, the cells were treated with **DOX-TCO** (0.2 μM) for 48 h. This concentration corresponds to the IC_{80} value of the prodrug. Meanwhile, complete conversion of the prodrug to **DOX** would correspond to the IC_{20} value of the latter. Thus, the activated prodrug is expected to be considerably more cytotoxic. Fluorescence microscopy confirmed that **MNP-cy5.5-tet**, as well as **DOX-TCO** were taken up by cells (**Figure 5c**). After the prodrug treatment, the cells were grown for another 24 h in DMEM to evaluate the cytotoxicity of the activated prodrug.

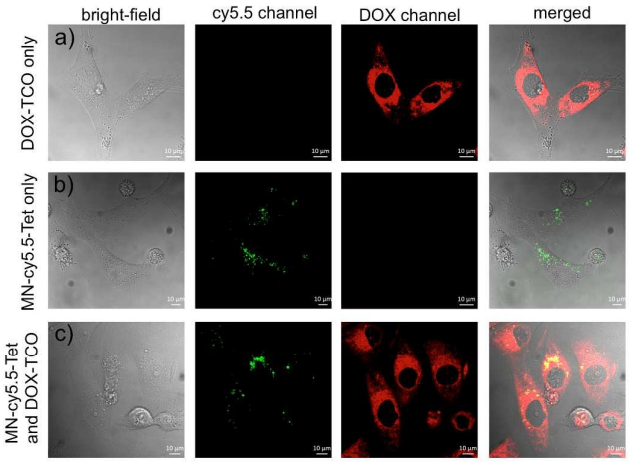

Figure 5. Confocal microscopy images of MDA-MB-231 breast cancer cells incubated with (a) **DOX-TCO** only, (b) **MNP-cy5.5-tet** only, and (c) **MNP-cy5.5-tet**, followed by **DOX-TCO**. The cy5.5 and **DOX-TCO** channels show nanoparticle and pro-drug uptake, respectively.

Additionally, flow cytometry (FACS) data indicated that almost all of the cells (99.9%) internalized **MNP-cy5.5-tet** (**Figure 6b**) and **DOX-TCO** (**Figure 6c**) when treated with each component separately. The negative control, shown in **Figure 6a** suggests that the cells without any treatment do not display any inherent cy5.5 or doxorubicin fluorescent signals. Finally, the FACS analysis of the cells treated with both **MNP-cy5.5-tet** and **DOX-TCO** exhibited fluorescent signals in both emission channels (**Figure 6d**).

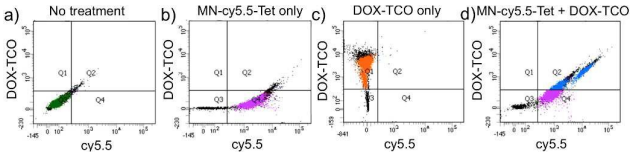

Figure 6. Flow cytometry histograms of MDA-MB-231 breast cancer cells incubated with (b) **MNP-cy5.5-tet** only, (c) **DOX-TCO** only, and (d) **MNP-cy5.5-tet**, followed by **DOX-TCO**.

Prodrug activation and ensuing release of **DOX** is expected to trigger apoptosis upon reaching cytotoxic concentrations inside the cells. Control experiments confirmed that **DOX-TCO** does not spontaneously get hydrolyzed into **DOX** in MDA-MB-231 cell lysate for as long as four days of incubation, **Figure S3**. The extent of intracellular activation of **DOX-TCO** by **MNP-cy5.5-tet** was assayed by measuring Caspase-3 activity indicative of apoptosis. **Figure 7a** illustrates the relative levels of Caspase-3 activity in cells treated with either **MNP-cy5.5-tet**, **DOX-TCO**, or the combination of the nanoparticles and the prodrug. Cells treated with **MNP-cy5.5-tet** alone showed minimal Caspase-3 activity, thus proving that the nanoparticles are not cytotoxic. Meanwhile, the combination therapy of **MNP-cy5.5-tet** and the prodrug showed activity analogous to the positive controls, supplied in the kit. The prodrug alone showed significantly lower Caspase-3 activity.

Cell viability assay (MTT) was carried out in parallel to confirm the findings of the Caspase-3 activity assay. As shown in **Figure 7b**, cells treated with **MNP-cy5.5-tet**, followed by **DOX-TCO** have similar levels of viability as the cells treated with **DOX**. Meanwhile, cells treated with either the nanoparticles or the prodrug alone have significantly higher levels of viability.

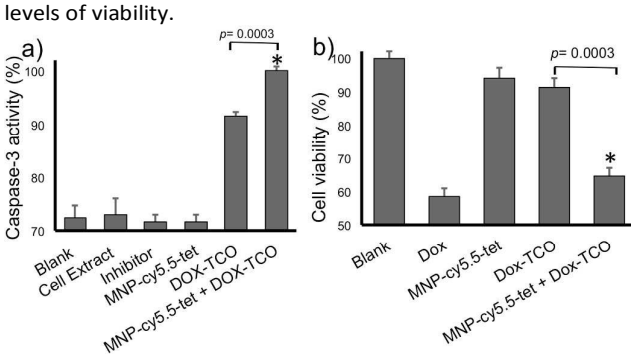

Figure 7. a) Caspase and b) cell viability assay (MTT) results demonstrate that the apoptosis of cells incubated with **MNP-cy5.5-tet** and **DOX-TCO** is significantly higher than the cells treated with **MNP-cy5.5-tet** and **DOX-TCO** separately.

Conclusions

In summary, a novel biomedical application of the bio-orthogonal 'click-to-release chemistry has been described. To our knowledge this is the first study describing multiplexing the therapeutic pontential of a chemotherapeutic prodrug using nanoparticles functionalized with tetrazines. The concept of image-guided *in situ* prodrug activation has been illustrated using TCO-modified doxorubicin. Meanwhile, tetrazine-functionalized MNPs served as image-guided triggers, capable of activating the prodrug inside of live breast cancer cells. As described above, this strategy has the potential for converting systemically administered prodrugs into image-guided targeted chemotherapeutics. Because of its modular nature, this methodology should be translatable to other chemotherapeutic agents modified with the releasable-TCO, as well as other nano-platforms.

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Footnote

M. Royzen and M. V. Yigit are corresponding authors. M. Royzen supervised the bioconjugation studies. M. V. Yigit supervised nanoparticle synthesis and characterization studies. Together they have designed the project, supervised *in vivo* studies, analyzed the data and wrote the paper. I. Khan performed all the experiments and helped in writing the paper.

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Notes and references

‡ The supporting information provides the details of **DOX-TCO** and **MNP-cy5.5-tet** syntheses. It also provides the details of the MTT and Caspase-3 activity assays.

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