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(54) **Title:** TETRAZINE AS A TRIGGER TO RELEASE CAGED CARGO

(57) **Abstract:** There is presented a kit comprising a masked active agent and a tetrazine trigger, the masked active agent comprising an active agent connected to a masking moiety comprising a monovinyl ether or an active agent enclosed within a cage moiety comprising a monovinyl ether or an allyl group, wherein the tetrazine trigger is configured to release the active agent from the masking moiety or the cage moiety. Methods of preparing the masked active agents, and uses of the kit are also presented.



Tetrazine as a Trigger to Release Caged Cargo

The invention relates to the field of small molecule chemical triggers and masked active agents and peptides, specifically, chemical triggers for specifically transforming a masked active agent into an active agent, or selectively unprotecting a peptide which work under physiological conditions.

Background of the Invention

The use of inactive agents that can be activated at a specific site is known in the art. For example, the use of a drug or therapeutic active agent in an inactive form, such as a prodrug, has been extensively studied. Such prodrug forms are used to enable an active agent to pass through the digestive system of a subject without degradation of the active agent, and to then be transformed in the gut or blood stream of the subject into the active form. Prodrug forms of therapeutic active agents have been activated in a targeted manner, by conjugating an activating enzyme to an antibody that targets tissue in the site of interest. Once the prodrug arrives at the target tissue, it is activated into the active form by the activating enzyme.¹

However, the activating enzyme must not be an endogenous enzyme, which would lead to non-specific activation of the prodrug, and as a result the activating enzyme is often highly immunogenic, making repeat administration impossible.²

In a further method of delivering and activating a therapeutic active agent to a target site of interest, the therapeutic active agent is coupled to a targeting moiety such as a monoclonal antibody. For example, this approach has been used to deliver anticancer therapeutics specifically to tumour cells in an attempt to mitigate toxicity of the therapeutic active agent to other cells of the body. Whilst this approach should reduce the side effects of such conjugated therapeutic active agents, it has met limited success as the coupled therapeutic active agents are often inactive or significantly less active in the coupled form, resulting in a loss of efficacy of the treatment, while the dosing levels are stoichiometric.

Methods of chemical activation of coupled active agents at a target site have been proposed in the art. For example, WO2012/156920 in the name of Koninklijke Philips Electronics N.V., discloses the use of tetrazines as chemical triggers to activate bio-orthogonal drugs comprising a trans-cyclooctene derivative. During the reaction with the tetrazine and the trans-cyclooctene, the strain in trans-cyclooctene ring is released, thereby driving the reaction forward.

However, coupling of the desired active agent to a trans-cyclooctene ring is synthetically complex, the resulting conjugated form of the active agent undergoes a significant background cleavage rate. In addition, further trans-cis isomerization of trans-cyclooctene has been shown to occur in the presence of catalysts such as free thiols or transition metals bound to serum proteins *in vivo*.³

Accordingly, one object of the present invention is the provision of an improved conjugated active agent and an improved method of activating the improved conjugated active agent at a specific site of interest.

Summary of the Invention

According to a first aspect of the invention there is provided a kit for the administration and activation of a masked active agent, the kit comprising a masked active agent and a trigger, the masked active agent comprising:

- i) an active agent connected to a masking moiety; or
- ii) an active agent enclosed within a cage moiety;

wherein the trigger is configured to release the active agent from the masking moiety or the cage moiety, and wherein the trigger is a tetrazine, and the masking moiety comprises a monovinyl ether and the cage moiety comprise a monovinyl ether or an allyl group.

Therefore, in some embodiments, the masked active agent comprises an active agent connected to a masking moiety, and the trigger is configured to initiate the controlled removal of the masking moiety from the active agent, wherein the masking moiety comprises a monovinyl ether and the trigger comprises a tetrazine.

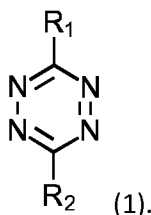
In other embodiments, the masked active agent comprises an active agent enclosed within a cage moiety, and the trigger is configured to release the active agent from the cage moiety, wherein the trigger is a tetrazine and the cage moiety comprises a monovinyl ether or an allyl group.

Typically, the allyl group may be an allyl ether, allyl ester or other activated allyl group.

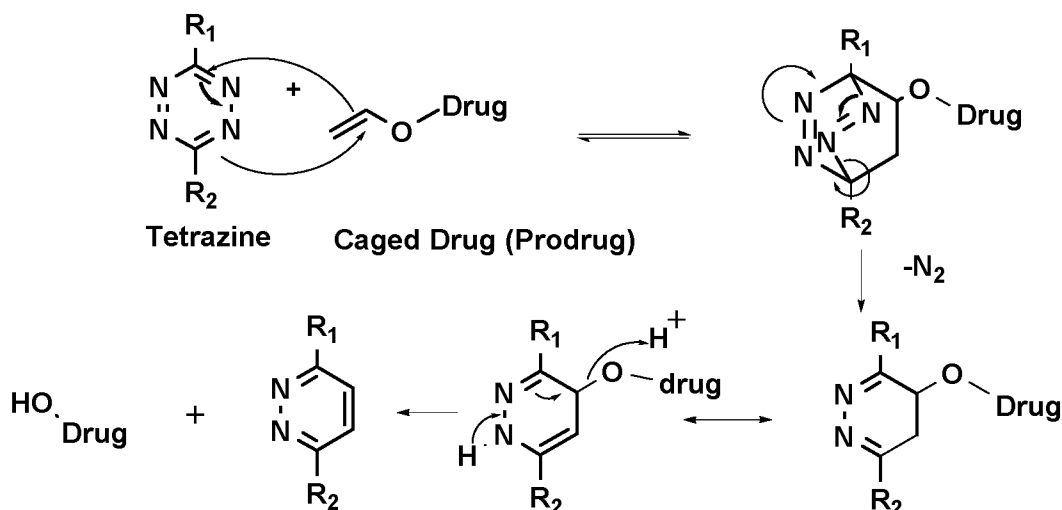
In some preferred embodiments, the masking moiety or the cage moiety comprise a phenolic monovinyl ether.

The kit of the invention allows selective release of an active agent from a masked active agent in a versatile way. The use of a masking moiety that comprises a monovinyl ether or cage moiety that comprises monovinyl ether or an allyl group allows a wide range of active agents to be converted to the masked form easily. For example, in embodiments where the masked active agent comprises a masking moiety, a hydroxyl group in the active agent may be converted to the monovinyl ether group. Accordingly, in some embodiments, the masked active agent is not significantly more bulky than the active agent itself, and may be formed readily from the active agent itself.

10 Preferably, the tetrazine has the general structure:



Typically, the reaction between the masked active agent and the tetrazine is an inverse electron demand Diels–Alder reaction, where the monovinyl ether group or allyl group acts as the dienophile, and the tetrazine acts as the diene. An example reaction scheme where the masked active agent comprises a monovinyl ether is shown in Scheme 1 below:



20 Scheme 1: Example reaction scheme of release of a drug (an example of an active agent) via an inverse electron demand Diels-Alder reaction.

The use of a tetrazine with a trans-cyclooctene moiety is known in the art. However, the masked active agent of the kit of the invention comprises a monovinyl ether or allyl group,

rather than a trans-cyclooctene ring moiety, which is typically simpler to synthesise from the active agent of interest, and results in the dienophile (the vinyl/ethylene group that reacts with the tetrazine) being more readily available for reaction.

- 5 In this inverse electron demand Diels-Alder reaction, the rate of reaction of a tetrazine with a vinyl group will typically depend in part on the identity of R_1 and R_2 . For example, if one or more of R_1 and R_2 are selected to be strong electron withdrawing groups, the electron density within the central tetrazine ring will be reduced, and the rate of reaction with a vinyl group may consequently increase. Alternatively, if one or more of R_1 and R_2 are selected to be weak
10 electron withdrawing groups, the electron density within the central tetrazine ring will be relatively higher, and the rate of reaction with a vinyl group may be consequently decreased.

Accordingly, the reactivity of the tetrazine with the masked active agent can be tailored by changing the identity of R_1 and/or R_2 . For example, if a slower rate of reaction is desired, R_1
15 and/or R_2 may be chosen to be weaker electron withdrawing groups, as defined below. In another example, if a faster rate of reaction is desired, R_1 and/or R_2 may be selected to be stronger electron withdrawing groups. However, R_1 and R_2 are typically selected to ensure that the resulting tetrazine is stable in aqueous solution.

- 20 In alternative embodiments, the tetrazine has the general structure (1) and one of R_1 and R_2 are chosen to be a polymer, and the remaining group R_1 and/or R_2 is chosen to be an electron withdrawing group or simple alkyl group. The polymer may act as a tethering group to bind the tetrazine to a specific area of interest or target tissue, for example, such that activation of the masked active agent predominantly occurs in the area of interest or in the target tissue.

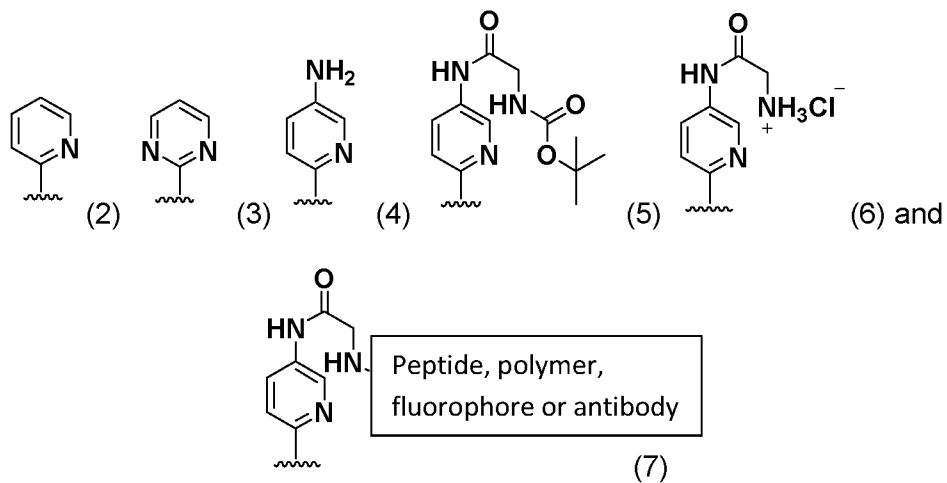
25 For example, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates and polyglutamate-paclitaxel display significant passive tumor targeting (>70-fold). This targeting may be due to the enhanced permeability and retention (EPR) effect.⁴

- 30 R_1 and R_2 may be weak electron withdrawing groups.

R_1 and R_2 may be weak electron withdrawing groups, and may be chosen from pyridines, diazines, aryl halides, aryl ketones, aryl aldehydes or aryl esters, such as methyl benzoate, for example.

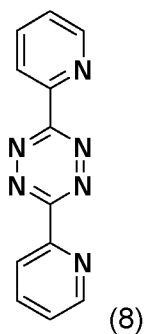
35 Preferably, R_1 and R_2 are selected from pyridines, and diazines.

Typically, R_1 and R_2 are independently selected from

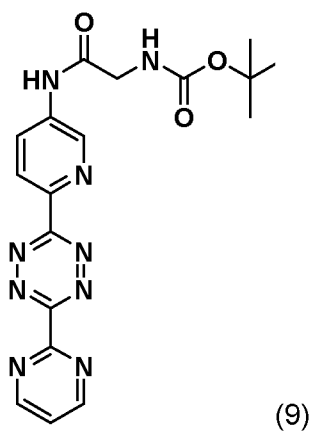


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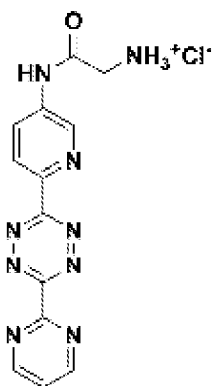
In one preferred embodiment of the invention, R_1 and R_2 are both (2) and the tetrazine has the structure (8):



10 In another preferred embodiment of the invention, R_1 is (5) and R_2 is (3) and the tetrazine has the structure (9):

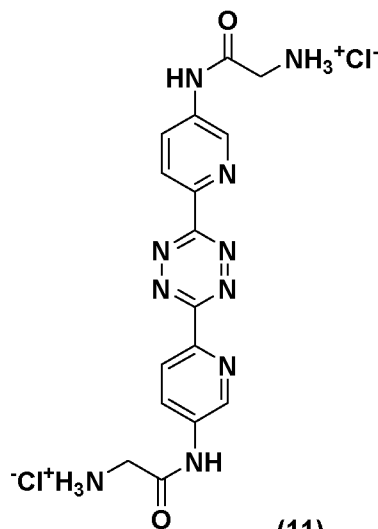


15 In a further preferred embodiment of the invention, R_1 is (6) and R_2 is (3) and the tetrazine has the structure (10):



(10)

In a further preferred embodiment of the invention, R₁ is (6) and R₂ is (6) and the tetrazine has the structure (11):



(11)

Tetrazines having the structures (8), (9), (10) and (11) have been found to be particularly effective as triggers for releasing active agents from masked active agents comprising monovinyl ether moieties or allyl group moieties under physiological conditions.

- 10 Preferably, the masked active agents are not triggered by endogenous species found in the environment within which the masked active agent is to be administered. For example, in
embodiments where the masked active agent is to be administered into a living subject, the
masked active agent is typically not triggered to release the active agent by enzymes, proteins
or chemical species that are present in the living subject. Accordingly, the active agent is
15 substantially only released when the trigger is administered and the trigger reacts with the
masked active agent, thereby providing specific and controllable release of the active agent
into the environment into which it has been administered.

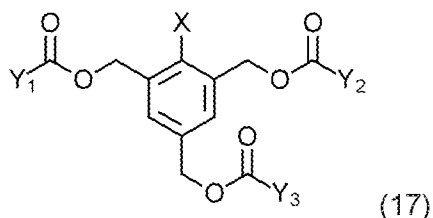
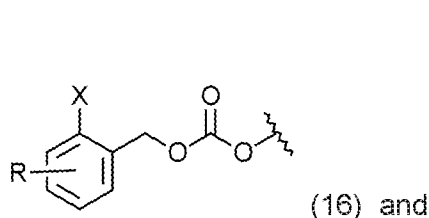
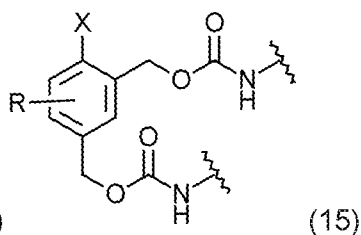
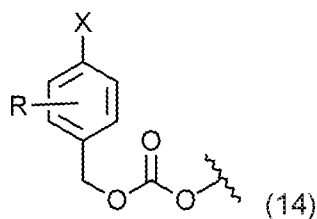
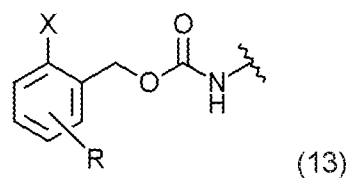
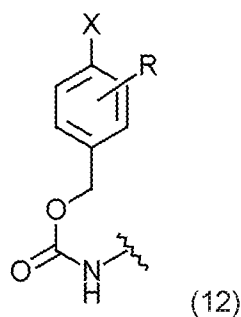
In some embodiments of the invention where the masked active agent comprises an active agent connected to a masking moiety, the active agent is directly linked to the masking moiety. Typically, the active agent comprises a hydroxyl group and the oxygen of the hydroxyl group of the active agent is connected to a vinyl group to thereby form the monovinyl ether masking moiety. Accordingly, when the masked active agent is triggered by the application of a tetrazine, the monovinyl ether is converted by reaction with the tetrazine to a hydroxyl group, thereby reforming the original active agent.

In alternative embodiments of the invention where the masked active agent comprises an active agent connected to a masking moiety, the active agent is indirectly linked to the masking moiety via a linker. This combination is known as a "safety catch" linker or protecting group which is tetrazine cleavable. Typically, the linker comprises a hydroxyl group and the oxygen of the hydroxyl group of the linker is chemically bonded to a vinyl group to thereby form the monovinyl ether masking moiety. The linker is also chemically bonded to the oxygen of the hydroxyl group of the active agent. The structure of the linker is chosen such that when the monovinyl ether is converted into a hydroxyl group on the linker, the newly formed hydroxyl group of the linker initiates a structural rearrangement reaction of the linker, resulting in the release of the active agent. For example, in embodiments where the linker comprises a phenol and a leaving group comprising the active agent at the 4- and/or 6- position, the structural rearrangement reaction may be a 1,4- and/or a 1,6-elimination reaction.^{5,6}

In some embodiments of the invention where the masked active agent comprises an active agent connected to a masking moiety and a linker, the linker may comprise a polymer. The polymer may be configured as a tether for the masked active agent via the linker, acting as an anchor for the masked active agent during use to fix the masked active agent to a specified location. The linker may comprise a bridge and a polymer extending away from the bridge. The bridge may connect the active agent to the masking moiety. The polymer may act as a targeting moiety such that the masked active agent preferentially targets or binds to a specific target, e.g. target tissue within which it is desired to activate the active agent.

Multiple bridges may be connected to the polymer such that multiple masked active agents are connected to a common polymer.

Preferably, the linker is selected from the group of linkers comprising:



wherein X is a monovinyl ether masking moiety;

Y₁, Y₂ and Y₃ are active agents;

10 and R is selected from H, C₁-C₂₀ alkyl, C₁-C₂₀ alkenyl, C₁-C₂₀ alkynyl, a polymer such as polyethylene glycol, poly(methyl methacrylate), poly(oxazoline), C₁-C₂₀ hydroxyl, halide (e.g. F, Cl, Br, I), C₁-C₂₀ ether, C₁-C₂₀ carboxylic acid, C₁-C₂₀ amide, C₁-C₂₀ amine, C₁-C₂₀ aldehyde, C₁-C₂₀ ketone.

15 In some embodiments, R may be selected from H, C₁-C₂₀ alkyl, C₁-C₂₀ alkenyl, C₁-C₂₀ alkynyl, a polymer such as polyethylene glycol, poly(methyl methacrylate), and poly(oxazoline).

For the avoidance of doubt, the active agent is connected to the carbamate group in structures (12), (13) and (15), to the carbonate group in structures (14) and (16), and to the carboxyl group in structure (17). Suitably, the active agent may be released from the masking moiety linked to the active agent via the linker (12) to (17) via a 1,4- and/or a 1,6-elimination reaction, respectively, when X is converted from a monovinyl ether to a hydroxyl group.

In embodiments where R is a polymer, the remainder of the linker may act as a bridge between the masking moiety and the active agent.

Typically, in embodiments where R comprises a moiety that is not H, R is connected to the benzene ring in (12) to (17) at the 3- or 5- position (meta position). Accordingly, the identity of R is less likely to interfere with the intended 1,4- and/or 1,6-elimination reaction that results in the release of the active agent.

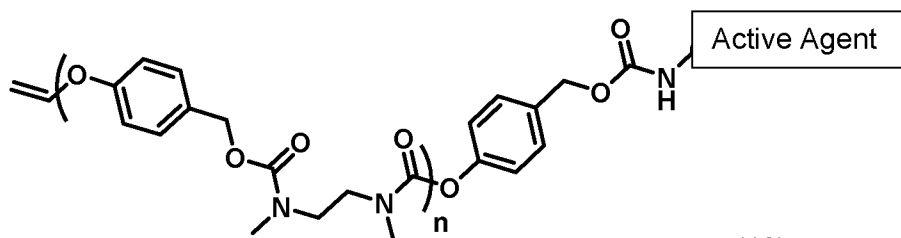
In some embodiments, R may be a polymer. Typically the polymer is a straight chain polymer and the active agent may be connected to a first end of the polymer and the masking moiety may be connected to the opposed second end of the polymer. However, the polymer may be a branched polymer and the active agent may be connected to a first end of the polymer and the masking moiety may be connected to the end of a branch of the polymer, or at the opposed end of the polymer chain.

In alternative embodiments, the polymer may comprise multiple copies of an active agent. The multiple copies of the active agent may be pendent from the polymer. In embodiments where the polymer is a straight chain polymer, the multiple copies of the active agent may be connected to the polymer periodically along the chain of the polymer. For example, a copy of the active agent may be connected to the polymer at every monomer subunit of the polymer, or at every other monomer subunit, or one active agent per three monomer subunits of the polymer etc. In embodiments where the polymer is a branched polymer, the multiple copies of the active agent may be located on one or more branches. Fragmentation of the polymer may release the multiple copies of the active agent from the polymer.

In another embodiment, the polymer chain may comprise multiple copies of the active agent. Accordingly, when the polymer fragments upon activation by a tetrazine, the fragments of the polymer may correspond to individual or multiple copies of the active agent. The fragments of the polymer may comprise individual or multiple copies of the active agent.

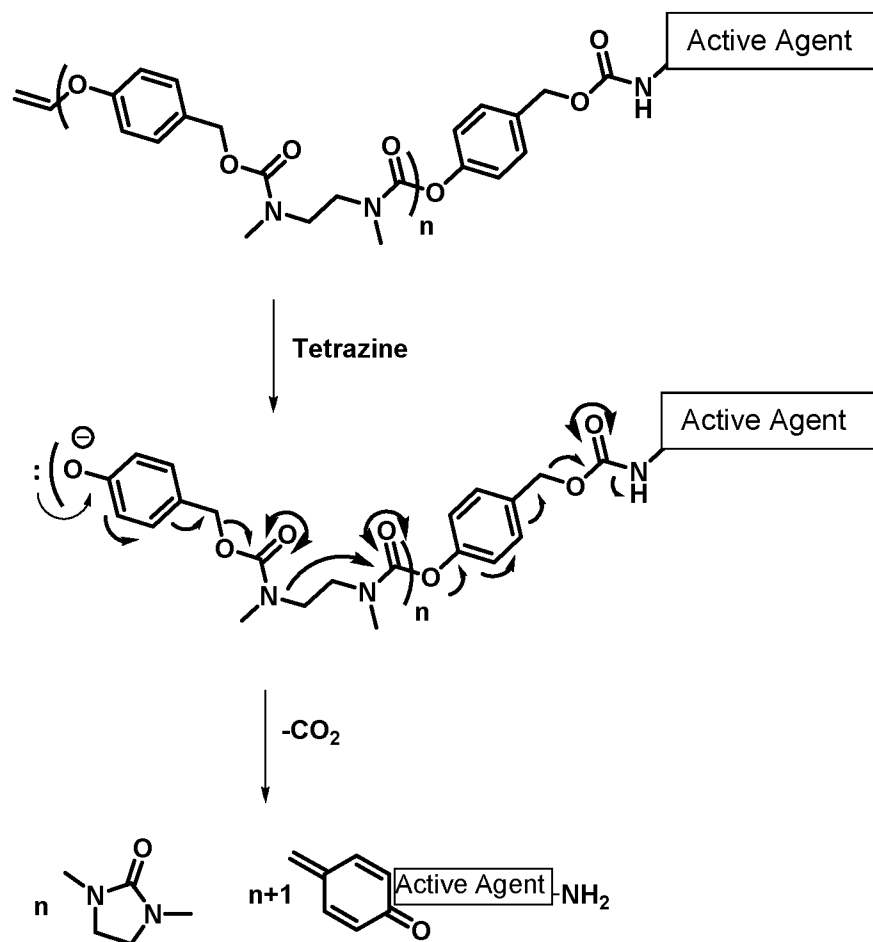
Preferably, the polymer is configured to fragment when the monovinyl ether moiety is removed from the polymer, to thereby release the active agent. The polymer may be configured to fragment via a cascade reaction when the monovinyl ether moiety is removed from the polymer.

For example, the polymer may have the structure:



(18)

and the polymer may fragment following removal of the monovinyl ether moiety via the general reaction as shown in scheme 2 below.



Scheme 2: fragmentation of an example polymer upon removal of the masking moiety.

Accordingly, the polymer may act as a spacer between the active agent and the monovinyl ether, or may act as a targeting moiety to direct binding of the masked active agent to a specific target site or target tissue, for example, such that when the masked active agent is triggered by a tetrazine trigger, the active agent is substantially only released in the target site of target tissue.

Typically, the monovinyl ether is removed from the masked active agent at room temperature. Preferably, the monovinyl ether is removed from the masked active agent in physiological conditions.

5 Accordingly, the kit is typically used in aqueous solutions at 37°C, conditions that are compatible with biological applications. For example, the kit may be used to administer a masked active agent to a subject and subsequent delivery of a tetrazine trigger may allow controlled release of the active agent within the subject. The masked active agent may be administered to a specific tissue or area of the subject and the trigger may be delivered
10 generally to the subject, activating the localised masked active agent in the specific tissue or area of interest. Alternatively, the masked active agent may be administered to a subject generally and the trigger may be administered to a specific tissue or target area of the subject such that only active agent in the specific tissue or target area of the subject are released by the trigger.

15 Some known methods of tetrazine triggered release of agents typically require elevated temperatures, and/or organic solvents that are not compatible with biological applications.

Therefore, the provision of a kit that allows tetrazine triggered release of active agents at room
20 temperature, and/or in physiological conditions, allows masked active agents to be used with biological samples *in vitro*, or *in vivo* within a subject.

In a further embodiment of the invention, the masked active agent may comprise a targeting moiety. The targeting moiety may preferentially bind to a specific target such that generally
25 administered masked active agent may tend to accumulate in or bind to the specific target. Accordingly, general or targeted administration of the trigger to the subject may predominantly release the active agent in the specific target area only. Therefore, the kit of the invention may allow specific delivery of the active agent to a specific target within the body of a subject.

30 In embodiments where the masked active agent comprises a masking moiety and the masking moiety comprises a polymer, the polymer may be configured to act as a targeting moiety.

The masked active agent may be a masked fluorophore. The masked fluorophore may be substantially non-fluorescent, and the fluorophore may be fluorescent when released from the
35 masking moiety or cage moiety by the trigger. The masked fluorophore may be fluorescent and the released fluorophore may be more fluorescent than the masked fluorophore.

Accordingly, the release of fluorophore from the masking moiety or cage moiety may be accompanied by an increase in fluorescence.

Typically, in embodiments where the masked fluorophore comprises a masking moiety, the masked fluorophore is a masked conjugated fluorophore.

By the term “conjugated fluorophore” we refer to a fluorophore comprising a conjugated pi system through which electrons are delocalised.

Suitably, the conjugated fluorophore comprises a hydroxyl or carbonyl group adjacent to the conjugated pi system, such that the masking moiety is directly bonded to the oxygen of the hydroxyl or carbonyl group of the conjugated fluorophore.

By the term “masked conjugated fluorophore” we refer to a conjugated fluorophore having a hydroxyl group that has been converted to a monovinyl ether. Typically, a masked conjugated fluorophore is substantially non-fluorescent or the level of fluorescence is significantly reduced compared to the fluorescence of the released conjugated fluorophore. Without wishing to be bound by theory, the inventors suggest that the reduction or inhibition of fluorescence may be due to the reduction in the extent of conjugation of the masked fluorophore due to the conversion of the hydroxyl group or carbonyl group to the monovinyl ether masking moiety.

For example, the masked fluorophore may be a masked fluorescein, a masked naphthofluorescein, a masked resorufin, a masked 5(6)-carboxyfluorescein or a masked coumarin.

The active agent may be a therapeutic active agent and the masked active agent may be a prodrug of that therapeutic active agent.

For example, the prodrug may be a monovinyl fluorouracil, a monovinyl doxorubicin, a monovinyl camptothecin, a monovinyl clofarabine, a monovinyl fludarabine, a monovinyl cladribine, a monovinyl etoposide, a monovinyl daunorubicin, or a monovinyl cis-platin. The prodrug of the therapeutic active agent may comprise a single masking moiety. The prodrug of the therapeutic active agent may comprise two masking moieties.

The therapeutic active agent may be cytotoxic. The prodrug of the cytotoxic therapeutic active agent may have reduced cytotoxicity. The prodrug of the cytotoxic therapeutic active agent may have substantially no cytotoxicity. Accordingly, targeted release of the active agent by a

tetrazine trigger *in situ* may allow the active agent to be cytotoxic at the target tissue or area of interest and not cytotoxic, or less cytotoxic in other tissues. Therefore, the kit of the invention may at least reduce the side effects associated with cytotoxicity affecting healthy cells.

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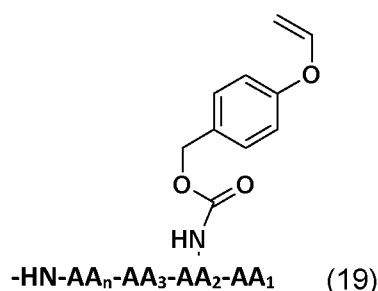
For example, a cytotoxic therapeutic active agent may target rapidly multiplying cells, and therefore, may target red blood cells in bone marrow and cells in the digestive tract, in addition to its intended target of tumour cells. Reducing the cytotoxicity of the therapeutic active agent by converting the therapeutic active agent to the prodrug form comprising a monovinyl ether, and activating the prodrug form with a tetrazine trigger at the site of the tumour or tumours to be treated may result in a reduction of side effects experienced by the subject, without compromising the potency of the therapeutic active agent.

Accordingly, the kit of the invention allows a prodrug form of a therapeutic active agent to be activated by release of the therapeutic active agent from the prodrug form of the active agent in the presence of tetrazine at the site of interest only.

The active agent may be a protein. The active agent may be a peptide or polypeptide. In embodiments where the masked active agent comprises a masking moiety, the masking moiety of the masked active agent may be bonded to the C- terminal of the peptide or protein. The masking moiety may be bonded to the N-terminal of the peptide or protein. The masking moiety may be bonded to an amino group of the peptide or protein.

For example, a masked peptide may have the general structure:

25



where AA_n is the nth amino acid in the sequence.

In embodiments where the masked active agent comprises a masking moiety, the masked active agent may be a masked protein or a masked peptide. For example, the monovinyl ether of the masking moiety may prevent a masked protein from folding correctly and thereby

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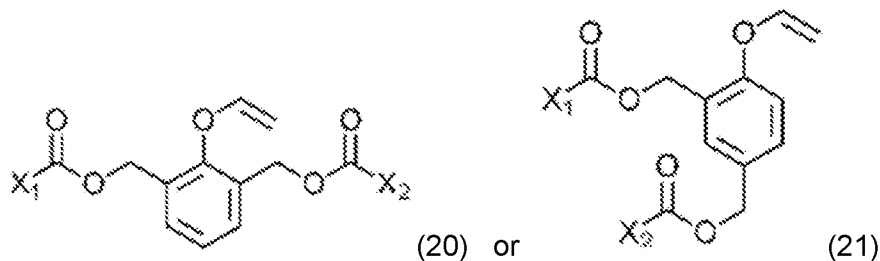
prevent or inhibit the functionality of the mis-folded protein. Removal of the masking moiety may allow the mis-folded protein of the masked protein to refold correctly and thereby restore the functionality of the protein.

- 5 In embodiments where the active agent is a peptide, the masked peptide may be more resistant to degradation of the peptide by enzymes in biological environments, and thereby allow the peptide to be released at the target where it is required, even in the presence of protease or the like. The masked peptide may allow selective peptide conjugation.
- 10 In embodiments where the masked active agent comprises a cage moiety, the cage moiety may encapsulate a protein, thereby sterically hindering the functionality of the encapsulated protein. Release of the protein from the cage moiety may therefore remove the steric hindrance to the protein and thereby restore the functionality of the protein.
- 15 Typically, in embodiments where the masked active agent comprises an active agent connected to a masking moiety, the masked active agent comprises at least one masking moiety. The masked active agent may comprise at least two masking moieties.

The masked active agent may be linked to a further active agent. In some embodiments, the further active agent may be active whilst linked to the masked active agent. For example, the further active agent may be a fluorophore that is fluorescent when linked to the masked active agent. Accordingly, masked active agent may be visualised during delivery to a target site during use.

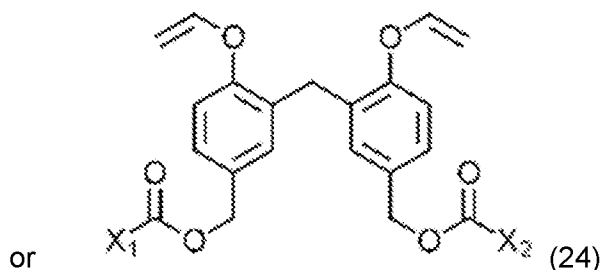
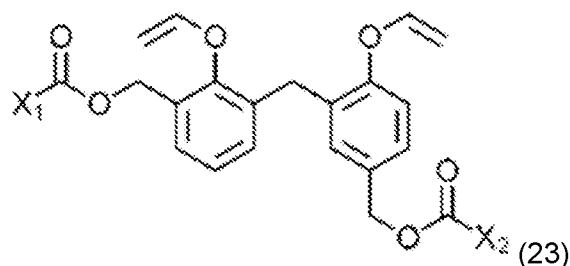
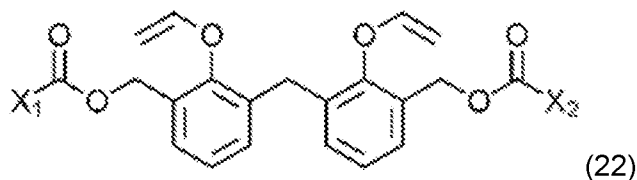
25 Alternatively, the further active agent may be a further masked active agent. The masked active agent may be linked to the further masked active agent via a common masking moiety, such that removal of the monovinyl ether of the masking moiety results in the release of the active agent and/or the further masked active agent.

30 In some embodiments, the common masking moiety may have the structure:



wherein X_1 is the active agent, and X_2 is the further active agent or vice versa.

In alternative embodiments where the masked active agent comprises at least two masking moieties comprising monovinyl ethers, the common masking moiety may have the structure:



wherein, X_1 is the active agent and X_2 is the further active agent.

For example, X_1 may be a therapeutic active agent and X_2 may be a fluorophore. Accordingly, the masked active agent may allow delivery and activation of a therapeutic active agent to a target site and at the same time allow visualisation of activation via fluorescence of the released fluorophore.

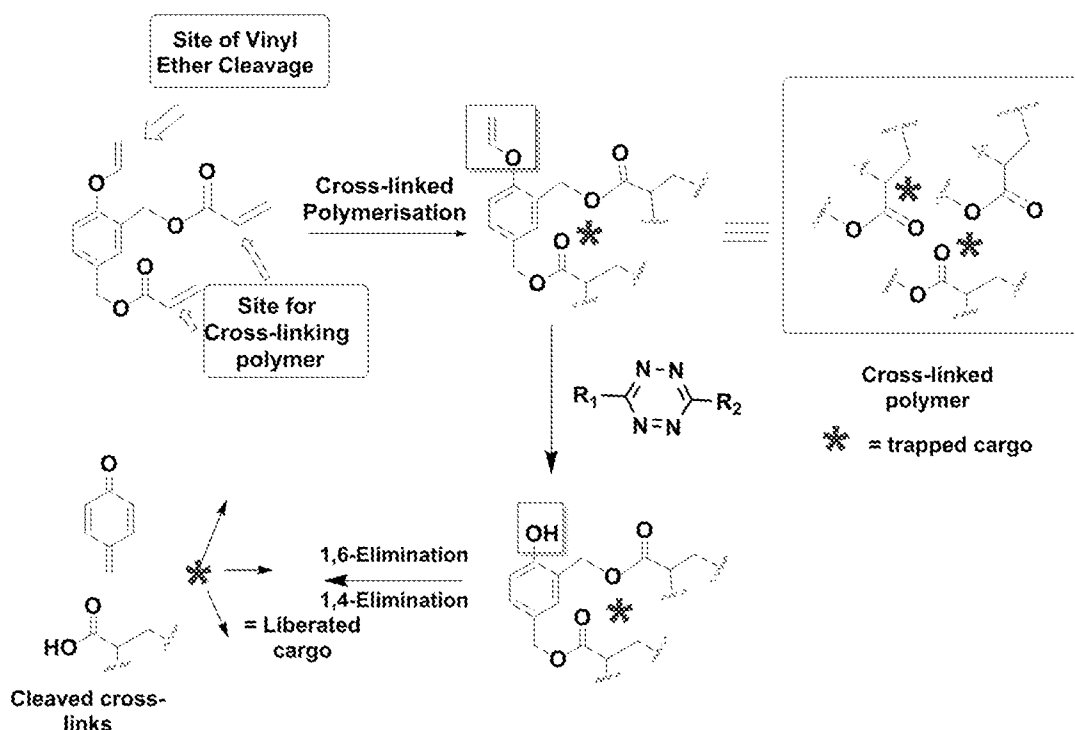
In an alternative example, X_1 and X_2 may be therapeutic active agents. X_1 and X_2 may be different therapeutic active agents. Therefore, the masked active agent may deliver two different therapeutic active agents to a target tissue or area of interest. X_1 and X_2 may be the same therapeutic active agent. Therefore, the masked active agent may deliver a higher concentration of the therapeutic active agent to the target tissue or area of interest.

In embodiments where the masked active agent comprises a cage moiety, the cage moiety may comprise a plurality of branch elements extending from a core. Typically, the core comprises a monovinyl ether, the removal of which results in the release of the active agent from the cage moiety. For example, the core may comprise a benzene ring and the oxygen of the monovinyl ether may be directly connected to the benzene ring, such that the trigger converts the aryl monovinyl ether to a phenol group that may then further react to release the active agent from the cage moiety. The phenol may allow one or more of the branch elements within the plurality of branch elements to be eliminated from the benzene ring via a 1,6- and/or 1,4-elimination pathway. The elimination of the one or more branch elements may allow the active agent to be released from the cage moiety.

In alternative embodiments, the core may comprise an allyl group and modification of the allyl group may result in the release of the active agent from the cage moiety.

At least some of the branch elements within the plurality of branch elements may be cross-linked such that the active agent is restrained between the plurality of branch elements, and therefore constitutes the masked active agent.

An example reaction scheme for the cross-linking polymerisation and subsequent release of the retained active agent via reaction with a tetrazine trigger is shown in scheme 3 below:

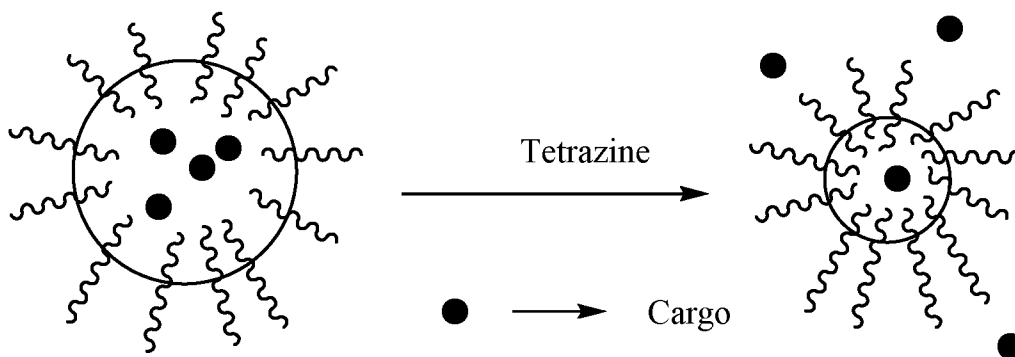


Scheme 3: cross-linking of cage moieties comprising monovinyl ether followed by reaction with a tetrazine trigger and subsequent release of the active agent (trapped cargo).

The masked active agent may comprise a plurality of cage moieties. The plurality of cage moieties may be linked. The plurality of linked cage moieties may form a superstructure that may encapsulate the active agent. The application of the trigger to the superstructure may initiate modification of the superstructure due to the reaction of the tetrazine with the monovinyl ether or allyl group of the plurality of cage moieties. For example, the superstructure may be modified sufficiently to release the active agent when the monovinyl ether or allyl group of at least one of the cage moieties react with the trigger. In another example, the superstructure may be modified sufficiently to release the active agent when the monovinyl ether or allyl group of the majority of the cage moieties react with the trigger. In a further example, the superstructure may be modified sufficiently to release the active agent when the monovinyl ether or allyl group of substantially all of the cage moieties react with the trigger.⁷

The plurality of cage moieties may comprise polymers. The polymers may form aggregates in aqueous solution. The aggregates may be nanoparticles in aqueous solution, such as micelles, or vesicles. Accordingly, an active agent may be encapsulated within the polymer nanoparticles in aqueous solution, for example.

The polymers may comprise one or more allyl groups. Accordingly, reaction of the allyl groups with a tetrazine may change the electrostatic charge on the polymer, thereby changing polymer's interaction with the aqueous solution. As a result, the shape or morphology of the polymer aggregates may change. For example, the size of the polymer aggregates may be reduced, thereby releasing at least a majority of an active agent retained therein (see Scheme 4 below).



Scheme 4 A change of physical properties of nanostructures (i.e. nanoparticles) can be triggered by tetrazine. A change in size leads to a release of cargo.

In another example, the morphology of the polymer aggregate may change such that the average spacing between the polymers making up the polymer aggregate increases, thereby allowing an active agent encapsulated within the polymer aggregate to be released.

5

In another example the superstructure may switch from hydrophobic to hydrophilic to release the active agent upon application of a tetrazine trigger.

The plurality of cage moieties may comprise one or more amino acids. The one or more amino acids of the plurality of cage moieties may form a shell around the active agent enclosed within the superstructure.

The superstructure may be a dendrimer structure. The dendrimer structure may comprise a plurality of generations terminating in cage moieties. Accordingly, the monovinyl ether or allyl groups of the cage moieties may be located at the perimeter of the dendrimer. For example, the superstructure may be a 5th generation poly(propyleneimine) dendrimer modified to include cage moieties at the terminus of each dendron.⁸

The superstructure may be in the form of nanoparticles, a hydrogel, fibres, vesicles or microcapsules.

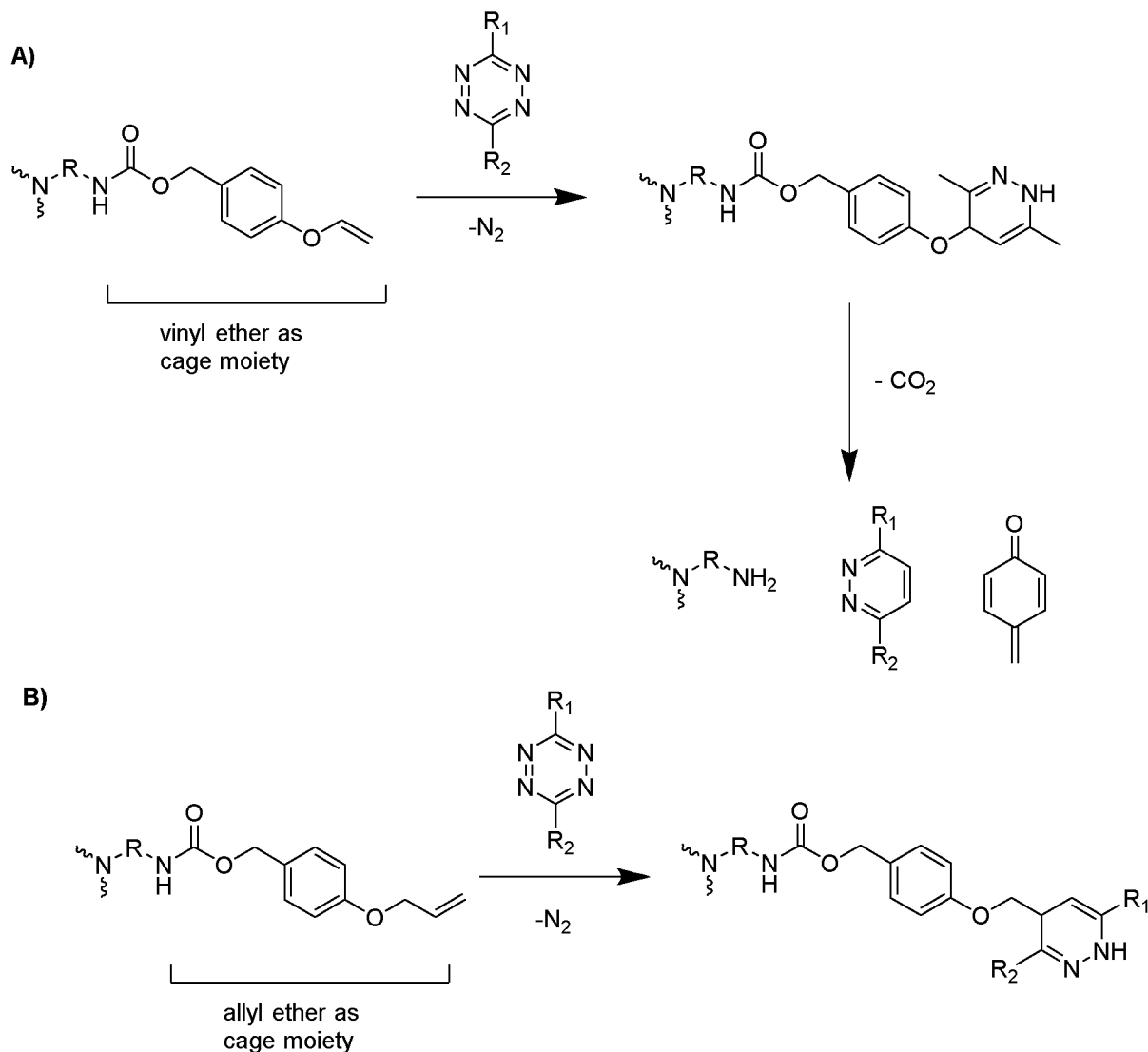
The active agent may be enclosed within the superstructure. Accordingly, the plurality of cage moieties may form a shell around the active agent. The plurality of cage moieties may form a superstructure around the active agent.

25

Release of the active agent from a masked active agent comprising a plurality of cage moieties forming a shell around the active agent may be triggered by reaction of the monovinyl ether moieties at the end of each cage moiety to result in primary amine groups, or by reaction of allyl group moieties to covalently attach a modifying group to the superstructure. These tetrazine triggered materials can be termed 'smart materials'

30

An example reaction scheme is shown in scheme 5 below.



Scheme 5 Vinyl ether (A) and allyl ether (B) as caging moieties. Vinyl ether (A) reacts with tetrazine to give a free amine whereas allyl ether (B) forms the dihydropyridazine. In both cases the nanostructure is converted from a hydrophobic into a hydrophilic one. For the avoidance of doubt, the N on the far left of the starting material is attached to superstructure, such as dendrons of a dendrimer, for example.

The invention extends in a second aspect to a masked active agent comprising an active agent connected to a masking moiety, or a masked active agent comprising an active agent enclosed within a cage moiety; wherein the masking moiety comprises a monovinyl ether and the cage moiety comprise a monovinyl ether or allyl group moiety.

Preferably, the masked active agent is configured to release the active agent from the masking moiety or the cage moiety in the presence of a trigger. Preferably, the trigger is a tetrazine.

The reaction of the tetrazine with the monovinyl ether of the masking moiety or the monovinyl ether or allyl ether of the cage moiety releases the active agent from the masking moiety or the cage moiety.

- 5 Typically, the masked active agent is substantially less active than the released active agent. In some embodiments, the masked active agent is substantially inactive and the released active agent is active.

10 In some embodiments of the invention where the masked active agent comprises a masking moiety and the active agent is connected to the masking moiety, the active agent is directly linked to the monovinyl ether moiety. Typically, the active agent comprises a hydroxyl group and the oxygen of the hydroxyl group of the active agent is chemically bonded to a vinyl group to thereby form the monovinyl ether masking moiety. Accordingly, when the masked active agent is triggered by the application of a tetrazine, the monovinyl ether is converted by reaction
15 with the tetrazine to a hydroxyl group, thereby reforming the original active agent.

In alternative embodiments of the invention where the masked active agent comprises a masking moiety, the active agent is indirectly linked to the masking moiety via a linker. Typically, the linker comprises a hydroxyl group and the oxygen of the hydroxyl group of the
20 linker is connected to a vinyl group to thereby form the monovinyl ether masking moiety. The linker may be connected to an oxygen of a hydroxyl group of the active agent. The structure of the linker is chosen such that when the monovinyl ether is converted into a hydroxyl group on the linker, the newly formed hydroxyl group of the linker initiates a structural rearrangement reaction of the linker, resulting in the release of the active agent. For example, in embodiments
25 where the linker comprises a phenol and a leaving group comprising the active agent at the 4- or 6- position, the structural rearrangement reaction may be a 1,4- or a 1,6-elimination reaction.

In some embodiments of the invention, the linker may comprise a polymer. The polymer may
30 be configured as a tether for the masked active agent via the linker, acting as an anchor for the masked active agent during use to fix the masked active agent to a specified location. The linker may comprise a bridge and a polymer extending away from the bridge. The bridge may connect the active agent to the masking moiety. The polymer may act as a targeting moiety such that the masked active agent preferentially targets or binds to a specific target, e.g. target
35 tissue within which it is desired to activate the active agent.

Preferably, the linker is selected from the group of linkers comprising structures (12) to (16) as defined in the first aspect.

For the avoidance of doubt, the active agent is connected to the carbamate group in structures (12) to (16). Suitably, the active agent may be released from the masking moiety linked to the active agent via the linker (12) to (16) via a 1,4- and/or a 1,6-elimination reaction, respectively.

In embodiments where R is a polymer, the remainder of the linker may act as a bridge between the masking moiety and the active agent.

Multiple bridges may be connected to the polymer such that multiple masked active agents are connected to a common polymer.

Typically, in embodiments where R comprises a moiety that is not H, R is connected to the benzene ring in (12) to (17) at the 3- or 5- position (meta position). Accordingly, the identity of R is less likely to interfere with the intended 1,4- and/or 1,6-elimination reaction that results in the release of the active agent.

In some embodiments, the linker may be a polymer. Typically the polymer is a straight chain polymer and the active agent may be connected to a first end of the polymer and the masking moiety may be connected to the opposed second end of the polymer. However, the polymer may be a branched polymer and the active agent may be connected to a first end of the polymer and the masking moiety may be connected to the end of a branch of the polymer, or at the opposed end of the polymer chain. Suitably, the polymer is configured to fragment when the monovinyl ether moiety is removed from the polymer, to thereby release the active agent.

For example, the polymer may have the structure (18) above, and fragment following removal of the monovinyl ether moiety via the general reaction as shown in scheme 2.

Accordingly, the polymer may act as a spacer between the active agent and the monovinyl ether, or may act as a targeting moiety to direct binding of the masked active agent to a specific target site or target tissue, for example, such that when the masked active agent is triggered by a tetrazine trigger during use, the active agent is substantially only released in the target site or target tissue.

Typically, the monovinyl ether is removed from the masked active agent at room temperature. Preferably, the monovinyl ether is removed from the masked active agent in physiological conditions.

5 Accordingly, the masked active agent may be triggered to release the active agent in aqueous solutions at 37°C, conditions that are compatible with biological applications. For example, the masked active agent may be administered to a subject and subsequent delivery of a tetrazine trigger may allow controlled release of the active agent within the subject. The masked active agent may be administered to a specific tissue or area of the subject and the
10 trigger may be delivered generally to the subject, activating the localised masked active agent in the specific tissue or area of interest. Alternatively, the masked active agent may be administered to a subject generally and the trigger may be administered to a specific tissue or target area of the subject such that only active agent in the specific tissue or target area of the subject are released by the trigger.

15 Some known methods of tetrazine triggered release of agents typically require elevated temperatures, and/or inorganic solvents that are not compatible with biological applications.

Therefore, the provision of a masked active agent that allows tetrazine triggered release of an
20 active agent from the masked active agent at room temperature, and/or in physiological conditions, allows masked active agents to be used with biological samples *in vitro*, or within a subject *in vivo*.

In a further embodiment of the invention, the masked active agent may comprise a targeting
25 moiety. The targeting moiety may preferentially bind to a specific target such that generally administered masked active agent may tend to accumulate in or bind to the specific target. Accordingly, general or targeted administration of the trigger to the subject may predominantly release the active agent in the specific target area only. Therefore, during use, the masked active agent may allow specific delivery of the active agent to a specific target within the body
30 of a subject.

In embodiments where the masked active agent comprises a masking moiety and the masking moiety comprises a polymer, the polymer may be configured to act as a targeting moiety.

35 The masked active agent may be a masked fluorophore. The masked fluorophore may be substantially non-fluorescent, and the fluorophore may be fluorescent when the masked fluorophore is released from the masking moiety or cage moiety by the trigger. The masked

fluorophore may be fluorescent and the released fluorophore may be more fluorescent than the masked fluorophore.

Accordingly, the release of fluorophore from the masking moiety or cage moiety may be accompanied by an increase in fluorescence.

Typically, in embodiments where the masked fluorophore comprises a masking moiety, the masked fluorophore is a masked conjugated fluorophore.

Suitably, the conjugated fluorophore comprises a hydroxyl or carbonyl group adjacent to the conjugated pi system, such that the masking moiety is directly bonded to the oxygen of the hydroxyl or carbonyl group of the conjugated fluorophore.

Typically, a masked conjugated fluorophore is substantially non-fluorescent or the level of fluorescence is significantly reduced compared to the fluorescence of the released conjugated fluorophore.

For example, the masked fluorophore may be a masked fluorescein, a masked naphthofluorescein, a masked resorufin, a masked 5(6)-carboxyfluorescein or a masked coumarin.

The active agent may be a therapeutic active agent and the masked active agent may be a prodrug of that therapeutic active agent.

For example, the prodrug may be a monovinyl fluorouracil, a monovinyl doxorubicin, a monovinyl clofarabine, a monovinyl fludarabine, a monovinyl cladribine, a monovinyl Etoposide, a monovinyl daunorubicin or a monovinyl camptothecin. The prodrug of the therapeutic active agent may comprise a single masking moiety. The prodrug of the therapeutic active agent may comprise two masking moieties.

Therapeutic active agents may be cytotoxic. The prodrug of the cytotoxic therapeutic active agent may have reduced cytotoxicity. The prodrug of the cytotoxic therapeutic active agent may have substantially no cytotoxicity. Accordingly, targeted release of the active agent by a tetrazine trigger *in situ* may allow the active agent to be cytotoxic at the target tissue or area of interest, and may therefore at least reduce the side effects associated with cytotoxicity affecting healthy cells.

For example, a cytotoxic therapeutic active agent may target rapidly multiplying cells, and therefore, may target red blood cells in bone marrow and cells in the digestive tract, in addition to its intended target of tumour cells. Reducing the cytotoxicity of the therapeutic active agent by converting the therapeutic active agent to the prodrug form comprising a monovinyl ether, and activating the prodrug form with a tertazine trigger at the site of the tumour or tumours to be treated may result in a reduction of side effects experienced by the subject, without compromising the potency of the therapeutic active agent.

Accordingly, the invention allows a prodrug form of a therapeutic active agent to be activated by release of the therapeutic active agent from the prodrug form of the active agent in the presence of tetrazine at the site of interest only.

The active agent may be a protein. The active agent may be a peptide or polypeptide. In embodiments where the masked active agent comprises a masking moiety, the masking moiety of the masked active agent may be bonded to the C- terminal of the peptide or protein. The masking moiety may be bonded to the N-terminal of the peptide or protein.

For example, a masked peptide may have the general structure (19).

In embodiments where the masked active agent comprises a masking moiety, the masked active agent may be a masked protein or a masked peptide. For example, the monovinyl ether of the masking moiety may prevent a masked protein from folding correctly and thereby prevent or inhibit the functionality of the mis-folded protein. Removal of the masking moiety may allow the mis-folded protein of the masked protein to refold correctly and thereby restore the functionality of the protein.

In embodiments where the active agent is a peptide, the masked peptide may prevent degradation of the peptide by enzymes in biological environments, and allow the peptide to be released at the target where it is required.

In embodiments where the masked active agent comprises a cage moiety, the cage moiety may encapsulate the protein, thereby sterically hindering the functionality of the encapsulated protein. Release of the protein from the cage moiety may therefore remove the steric hindrance to the protein and thereby restore the functionality of the protein.

Typically, in embodiments where the masked active agent comprises a masking moiety, the masked active agent comprises at least one masking moiety. The masked active agent may comprise at least two masking moieties.

- 5 The masked active agent may be linked to a further active agent. The further active agent may be a further masked active agent. The masked active agent may be linked to the further masked active agent via a common masking moiety, such that removal of the monovinyl ether of the masking moiety results in the release of the active agent and/or the further masked active agent.

10

Alternatively, the further active agent is a further masked active agent and the further masked active agent is inactive or less active when linked to the masked active agent via a common masking moiety.

- 15 In some embodiments, the common masking moiety may have the structure (20) or (21).

In alternative embodiments, the common masking moiety may have the structure (22) to (24).

- For example, X_1 may be a therapeutic active agent and X_2 may be a fluorophore. Accordingly,
20 the masked active agent may allow delivery and activation of a therapeutic active agent to a target site and at the same time allow visualisation of activation via fluorescence of the released fluorophore.

- In an alternative example, X_1 and X_2 may be therapeutic active agents. X_1 and X_2 may be
25 different therapeutic active agents. Therefore, the masked active agent may deliver two different therapeutic active agents to a target tissue or area of interest. X_1 and X_2 may be the same therapeutic active agent. Therefore, the masked active agent may deliver a higher concentration of the therapeutic active agent to the target tissue or area of interest.

- 30 In some embodiments, the further active agent may be active whilst linked to the masked active agent. For example, the further active agent may be a fluorophore that is fluorescent when linked to the masked active agent. Accordingly, masked active agent may be visualised during delivery to a target site during use.

- 35 In embodiments where the masked active agent comprises a cage moiety, the cage moiety may comprise a plurality of branch elements extending from a core. Typically, the core comprises a monovinyl ether or allyl group, the modification of which results in the release of

the active agent from the cage moiety. For example, the core may comprise an aryl monovinyl ether and the oxygen of the monovinyl ether may be directly connected to the benzene ring, such that the trigger converts the aryl monovinyl ether group to a phenol group that may then further react to release the active agent from the cage moiety. The phenol may allow one or more of the branch elements within the plurality of branch elements to be eliminated from the benzene ring of the phenol group via a 1,6- or 1,4-elimination pathway. The elimination of the one or more branch elements may allow the active agent to be released from the cage moiety.

In an alternative embodiment, the core may comprise an allyl group, modification of which converts the allyl group to a dihydropyridazine. Accordingly, the chemical and physical properties of the superstructure may be modified to allow release of the active agent from the masked active agent.

At least some of the branch elements within the plurality of branch elements may be cross-linked such that the active agent is restrained between the plurality of branch elements, and therefore, constitutes the masked active agent.

The masked active agent may comprise a plurality of cage moieties. The plurality of cage moieties may be linked. The plurality of linked cage moieties may form a superstructure that may encapsulate the active agent.

In embodiments where the caging moiety is a monovinyl ether, the application of the trigger to the superstructure may initiate fragmentation of the superstructure due to the reaction of the tetrazine with the monovinyl ether of the plurality of cage moieties. For example, the superstructure may fragment sufficiently to release the active agent when the monovinyl ether of at least one of the cage moieties react with the trigger. In another example, the superstructure may fragment sufficiently to release the active agent when the monovinyl ether of the majority of the cage moieties react with the trigger. In a further example, the superstructure may fragment sufficiently to release the active agent when the monovinyl ether of substantially all of the cage moieties react with the trigger.

In an alternative embodiment, the core may comprise an allyl group, modification of which converts the allyl group to a hydro pyridazine. Accordingly, the chemical and physical properties of the superstructure may be modified to allow release of the active agent from the masked active agent.

An example reaction scheme for the cross-linking polymerisation and subsequent release of the retained active agent via reaction with a tetrazine trigger is shown in scheme 3 above.

The plurality of cage moieties may comprise one or more amino acids. The one or more amino acids of the plurality of cage moieties may form a shell around the active agent enclosed within the superstructure.

The superstructure may form a dendrimer structure. The dendrimer structure may comprise a plurality of generations terminating in cage moieties. Accordingly, the monovinyl ether or allyl groups of the cage moieties may be located at the perimeter of the dendrimer. For example, the superstructure may be a 5th generation poly(propyleneimine) dendrimer modified to include cage moieties at the terminus of each dendron.

The active agent may be enclosed within the superstructure. Accordingly, the plurality of cage moieties may form a shell around the active agent. The plurality of cage moieties may form a superstructure around the active agent.

Release of the active agent from a masked active agent comprising a plurality of cage moieties forming a shell around the active agent may be triggered by reaction of the monovinyl ether or allyl group moieties at the end of each cage moiety.

An example reaction scheme is shown in scheme 5 above.

Preferred and optional features of the masked active agent of the first aspect are preferred and optional features of the second aspect.

In a third aspect of the invention, there is provided a pharmaceutical composition comprising a prodrug form of a therapeutic active agent, wherein the prodrug form of the therapeutic active agent comprises the therapeutic active agent and a monovinyl ether moiety.

The pharmaceutical composition may comprise a diluent. The pharmaceutical composition may comprise a filler. The pharmaceutical composition may comprise a carrier. The pharmaceutical composition may comprise an excipient. The pharmaceutical composition may comprise a lubricant or lubricating agent. The pharmaceutical composition may comprise a dispersing agent.

The prodrug may be configured to release the therapeutic active agent upon reaction of the prodrug with a tetrazine trigger.

Accordingly, the prodrug may be a prodrug according to any of the first and second aspects.

5

According to a fourth aspect of the invention there is provided a method of modifying an active agent into a masked active agent comprising the steps of:

- (a) providing an active agent; and
- (b) chemically linking the active agent to a vinyl group via a hydroxyl group of the active agent to form a monovinyl ether;

10

wherein the monovinyl ether is configured to be converted to a hydroxyl group by a tetrazine trigger.

The resulting masked active agent may be a masked active agent according to the second aspect of the invention, and may be provided in the kit of the first aspect of the invention.

15

According to a fifth aspect of the invention, there is provided a method of modifying an active agent into a masked active agent comprising the steps of:

- (a) providing an active agent; and
- (b) encapsulating the active agent with one or more caging moieties to form a masked active agent;

20

wherein the caging moiety comprises a monovinyl ether configured to be removed from the caging moiety by a tetrazine trigger, or

wherein the caging moiety comprises an allyl group configured to be modified by a tetrazine trigger,

25

such that the active agent may be released from the masked active agent upon application of a tetrazine trigger.

The resulting masked active agent may be a masked active agent according to the second aspect of the invention, and may be provided in the kit of the first aspect of the invention.

30

The invention extends in a sixth aspect to the use of a tetrazine as a trigger for the release, in a physiological environment, of an active agent, wherein the active agent is connected to one or more monovinyl ether moieties before release to form a masked active agent, and wherein the one or more monovinyl ether moieties are removed from the active agent by the tetrazine during release.

35

The active agent may be directly connected to the one or more monovinyl ether moieties. The active agent may be indirectly connected to the one or more monovinyl ether moieties via a linker.

- 5 Accordingly, the masked active agent may be a masked active agent according to the second aspect of the invention.

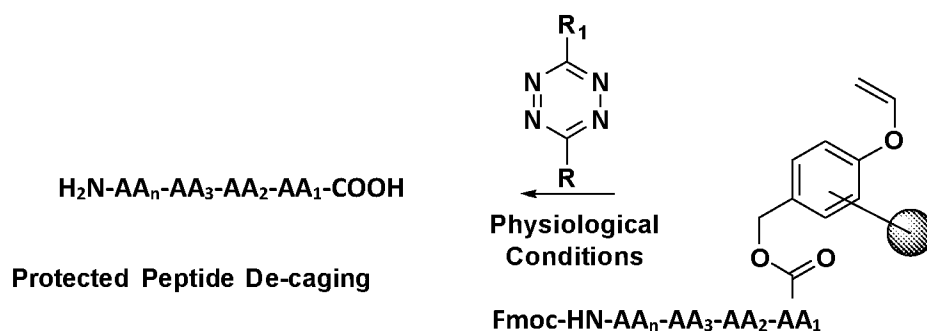
10 In a seventh aspect of the invention there is provided the use of a tetrazine to remove one or more monovinyl ether groups during active agent synthesis, wherein the one or more monovinyl ether groups prevent hydroxyl groups on the substrate from reacting in one or more earlier steps of the synthesis.

Accordingly, the one or more monovinyl ether groups may be acting as a protecting group during at least one step of the synthesis.

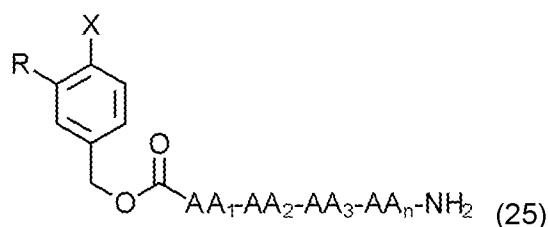
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In a eighth aspect, the invention extends to the use of a tetrazine to selectively remove one or more monovinyl ether groups from a peptide comprising one or more monovinyl ether groups.

20 The monovinyl ether group may act as a cleavable linker during synthesis of a peptide. An example of using a tetrazine trigger to cleave a linker comprising a monovinyl ether connecting a peptide to a support during synthesis of that peptide is shown in scheme 6 below.



25 Scheme 6 Synthesis of a peptide using the reaction between a tetrazine and a monovinyl ether to cleave the peptide from a support structure.



An example of a protected peptide is shown as structure (25), where X is monovinyl ether, and AA_n is the nth amino acid in the sequence of the peptide.

5 According to an ninth aspect of the invention, there is provided the use of the inverse electron-demand Diels-Alder reaction between a monovinyl ether and a tetrazine as a chemical tool for the release, in a physiological environment, of a substance administered as a masked active agent comprising a monovinyl ether.

10 According to a tenth aspect of the invention there is provided the use of a tetrazine as a trigger for polymer fragmentation for a polymer linked to a monovinyl ether.

The polymer may comprise an active agent. The polymer may have a linear structure. The active agent may be linked to the polymer at a first end of the polymer and the monovinyl ether
15 may be linked to a second opposed end of the polymer. The polymer may have a branched structure. The active agent may be linked to one of the branches of the polymer and the monovinyl ether may be linked to another branch of the polymer.

The polymer may encapsulate an active agent and fragmentation of the polymer activated by
20 the reaction of the tetrazine with the monovinyl ether may release the active agent.

The polymer may form aggregates in aqueous media and the active agent may be encapsulated within the aggregates. Tetrazine triggered fragmentation of the polymers that make up the aggregates may release the active agent therein.

25 Fragmentation of the polymer activated by the reaction of the tetrazine with the monovinyl ether may release the active agent. The polymer comprising the active agent may correspond to a masked active agent in accordance with the first and second aspects of the invention. Accordingly, the polymer may have the general structure (18).

30 Preferred and optional features of the polymer and the fragmentation of the polymer of the first aspect are preferred and optional features of the ninth aspect.

In a further aspect the invention extends to the use of a tetrazine as an activator for modifying
35 the hydrophobicity of a polymer comprising one or more allyl groups.

The polymer may be hydrophobic, or substantially hydrophobic, and the reaction of a tetrazine with the one or more allyl groups of the polymer may increase the hydrophilicity of the polymer, thereby modifying the hydrophobicity of the polymer.

- 5 Preferably, at least one of the monomers of the polymer comprises one or more allyl groups.

Preferably, the tetrazine comprises one or more hydrophilic groups.

- 10 The polymer may form aggregates in aqueous solution and modification of the hydrophobicity of the polymer may result in a change in the morphology of the aggregates such that active agents encapsulated within the aggregates before activation by the tetrazine are released.

Preferred and optional features of the polymer and reaction of tetrazines with allyl groups of the first aspect are preferred and optional features of the present aspect.

15

Brief Description of the Figures

Embodiments of the present invention will now be described, by way of non-limiting example, with reference to the accompanying drawings.

20

Figure 1. a). Structures of bis-O-vinylfluorescein and fluorescein. b). Fluorescence intensity of Fluorescein and bis-O-Vinylfluorescein at 2 μ M;

- Figure 2.** Graph showing percentage of bis-O-vinylfluorescein remaining and converting to mono-vinylfluorescein before converting to fluorescein. Calculated by using ^1H NMR via decrease in integration of vinyl protons in comparison to standard (1,2,4,5 Tetramethylbenzene);
- 25

Figure 3. ^1H NMR spectra of vinyl protons decreasing over time;

- Figure 4.** HPLC chromatogram showing conversion of vinyl ether to free hydroxyl group via TZ3. At T= 0 hrs no fluorescein is present but after T = 24 hrs fluorescein peak appears with presence of mono-vinylfluorescein and some bis-O-vinylfluorescein still remaining;
- 30

Figure 5. HPLC chromatogram showing calculated area after 24 hrs of fluorescein mono-vinylfluorescein and bis-O-vinylfluorescein;

Figure 6. Graph showing increase in fluorescence in comparison to bis-O-vinylfluorescein over time indicating release of fluorescein;

- 35 **Figure 7.** Graph showing 20 fold increase in fluorescence in comparison to bis-O-vinylfluorescein over time indicating release of fluorescein;

Figure 8. Graph showing increase in fluorescence in comparison to bis-O-vinylfluorescein over time indicating release of fluorescein;

Figure 9. Graph showing 123 fold increase in fluorescence in comparison to bis-O-vinylfluorescein over time indicating release of fluorescein;

5 **Figure 10.** a) Structures of vinylresorufin and resorufin. b) Fluorescence intensity of resorufin and vinylresorufin at 2 μ M;

Figure 11. Graph showing percentage of vinylresorufin remaining and converting to resorufin. Calculated by using ^1H NMR via decrease in integration of vinyl protons in comparison to standard (1,2,4,5 Tetramethylbenzene);

10 **Figure 12.** Graph showing percentage of vinylresorufin remaining. Calculated by using ^1H NMR via decrease in integration of vinyl protons in comparison to standard (1,2,4,5 Tetramethylbenzene);

Figure 13. ^1H NMR spectra of vinyl protons decreasing over time;

Figure 14. HPLC chromatogram showing conversion of vinyl ether to free hydroxyl group via TZ3. At T= 0 hrs no resorufin is present but after T = 24 hrs resorufin peak appears with vinylresorufin still remaining;

Figure 15. HPLC chromatogram showing calculated area after 24 hrs of TZ3, vinylresorufin and resorufin;

Figure 16. Graph showing increase in fluorescence in comparison to vinylresorufin over time indicating release of resorufin (TZ1);

Figure 17. Graph showing 32.6 fold increase in fluorescence in comparison to vinylresorufin over time indicating release of resorufin (TZ1);

Figure 18. Graph showing increase in fluorescence in comparison to vinylresorufin over time indicating release of resorufin (TZ3);

25 **Figure 19.** Graph showing 10.8 fold increase in fluorescence in comparison to vinylresorufin over time indicating release of resorufin (TZ3);

Figure 20. Graph showing cell viability of TZ1 and TZ3;

Figure 21. Graph showing cell viability of Vinylresorufin (VR), Vinylfluorescein (VF), Fluorescein (F) and Resorufin (R);

30 **Figure 22.** Graph showing percentage of vinyl groups of Divinyl-5, Fluorouracil remaining. Calculated by using ^1H NMR via decrease in integration of vinyl protons in comparison to standard (1,2,4,5 Tetramethylbenzene). c). ^1H NMR spectra of vinyl protons decreasing over time;

Figure 23. Graph showing percentage of vinyl group of polymer P1 reacted with tetrazine TZ1 at 37 °C. Calculated by using ^1H NMR via decrease in integration of vinyl protons in comparison to standard (1,2,4,5 Tetramethylbenzene);

Figure 24. ^1H -NMR of PAGE homopolymer P3 (9k Da) and tetrazine (9) in d_6 -DMSO at 60°C . The NMR was measured every 30 min;

Figure 25 Conversion of reaction between P3 (PAGE homopolymer, 9 kDa) and tetrazine (9) at different temperatures in d_6 -DMSO. Round = 100°C , triangle = 60°C and square = 25°C ;

5 **Figure 26.** Conversion of reaction between polymer with different molecular weight and tetrazine 9 at 60°C in d_6 -DMSO. Round = 1 kDa, triangle = 9 kDa and square = 40 kDa;

Figure 27. A) Size of nanoparticles formed by PEG-co-PAGE (P6) measured by DLS before tetrazine was added (line) and 24 h after tetrazine was added (dots). B) Tetrazine 11 C) TEM images before tetrazine 11 was added (a) and 24 h after tetrazine 1 was added (b);

10 **Figure 28.** Release profile of encapsulated Rhodamine B in nanoparticles formed by block PEG-co-PAGE (P6) in water. The experiment was carried out in water at 37°C and followed for 72 hours by fluorescence detection. Tetrazine 11 (TZ37) was added after 210 min. The black line shows the control experiment where no tetrazine was added whereas the red line shows the experiment which includes the addition of tetrazine;

15 **Figure 29.** Synthesis and characterisation of Doxorubicin conjugated nanoparticles (PEG-*b*-Dox). a) i) Doxorubicin hydrochloride, Et_3N , 51%; ii) PEG CTA, APS, TMEDA, DMSO, 30°C . b) Release profile of Dox from monomer **14** (2.3 mM) by tetrazine TZ1 (23 mM) at 37°C in PBS/ACN as monitored by HPLC (λ_{Abs} 495 nm). c) Size analysis (by dynamic light scattering) of the PEG-*b*-Dox derived nanoparticles showing an average diameter of 35 nm in PBS (pH
20 7.4) at 37°C ;

Figure 30. Tetrazine triggered release of Doxorubicin. HEK273T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. Nanoparticles, tetrazine **5** and/or free Doxorubicin were incubated with cells at 37°C with 5% CO_2 for 48 h. a) Control (just cells); b) PEG-*b*-Dox **15** nanoparticles (1 μM equiv. of Dox); c) Tetrazine **5** (35
25 μM); d) Tetrazine **5** (35 μM) and PEG-*b*-Dox **15** nanoparticles (1 μM). The samples were stained with propidium iodide (2 μM) and analysed by flow cytometry (λ_{Ex} 488 nm with 500–554 nm broad pass filter). Forward versus side scatter (SSC-A) profiles were used to gate intact cellular materials and determine membrane integrity (PI);

Figure 31. The block co-polymer PEG-*b*-PAGE (M_n = 5.8 kDa; PDI = 1.03) forms core-shell
30 nanoarchitectures in water. Modification of the allyl ether groups on the polymer backbone with a cationic tetrazine through an inverse electron demand Diels-Alder reaction resulted in the generation of NP micelles much reduced in size compared to the original vesicles. The morphologies were confirmed by TEM (staining with uranyl acetate) showing in a) vesicles in the absence of tetrazine. b) shows a single vesicle with the membrane thickness indicated by
35 arrows (~ 4 nm). c) micelles in the presence of tetrazine. d) shows a single micelle with diameter ≈ 30 nm. Scale bars represent 200 nm for a) and c), 50 nm for b) and d). f) The chemical structure of the PEG-*b*-PAGE used;

Figure 32. a) Size change and b) Release profiles with and without tetrazine (TZ) trigger of vesicles. Error bars represent standard deviations (n=3); and

Figure 33. PC3 cells were stained with CellTracker™ Green prior to incubation with NPs which were loaded with DOX (at 1 μ M, 6 μ M and 18 μ M) at 37 °C in the presence of 5% CO₂ for 72 hours. (a-b) Cells were harvested, stained with propidium iodide PI (1 μ g ml⁻¹) and examined by flow cytometry. The x-axis measures the fluorescein intensity of cells (FITC) due to CellTracker™ staining, (cell viability) and the y-axis corresponds to the PI fluorescence intensity due to the dead cell staining. Forward versus Side scatter profiles were used to gate-in intact cellular materials (remove cell debris) and determine the live cells and dead cells of each cell (see supporting information). Quadrant regions were derived from the gate and set to delineate the FITC and PI population. a) Without and b) with tetrazine triggering for different concentration of DOX loaded NPs. c) summary of cell viability in the presence of different concentration of DOX loaded NPs (errors are the standard deviation from the means, n =3).

Detailed Description

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

Vinyl ether caged fluorophore has been designed, synthesized and characterized. The release of the corresponding fluorophores by tetrazines can be detected by means of a suitable technique (HPLC, NMR, and Fluorescence Detection).

Materials and Methods

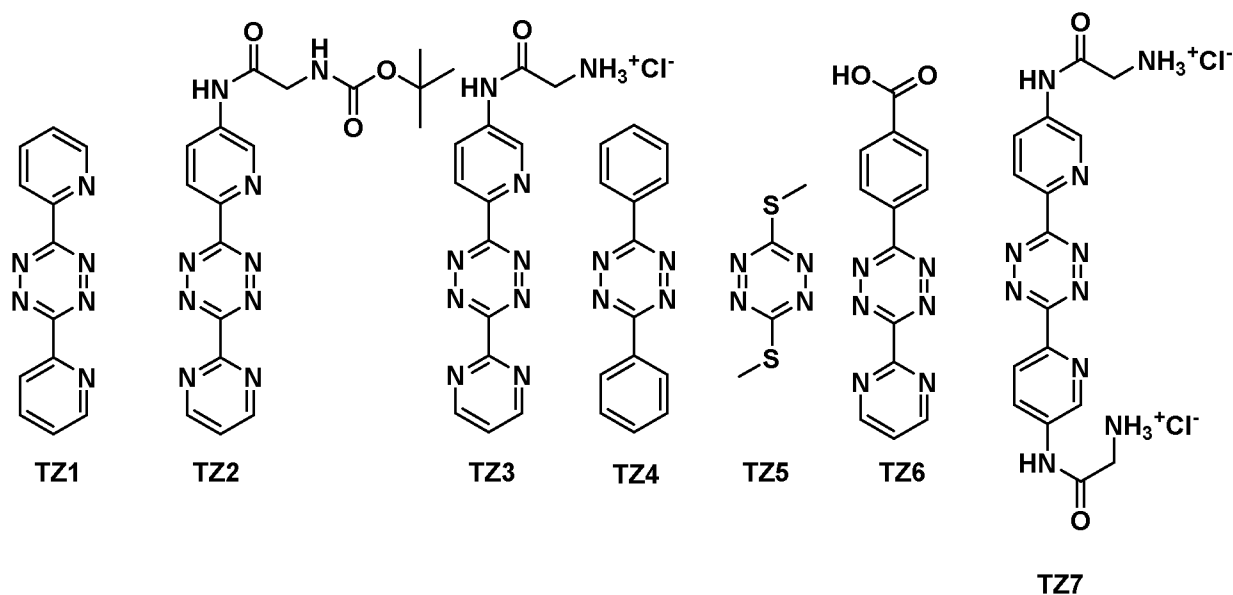
General

Commercially available reagents were used without further purification. NMR spectra were recorded using Bruker AC spectrometers operating at 500MHz or 600 MHz for ¹H and ¹³C.

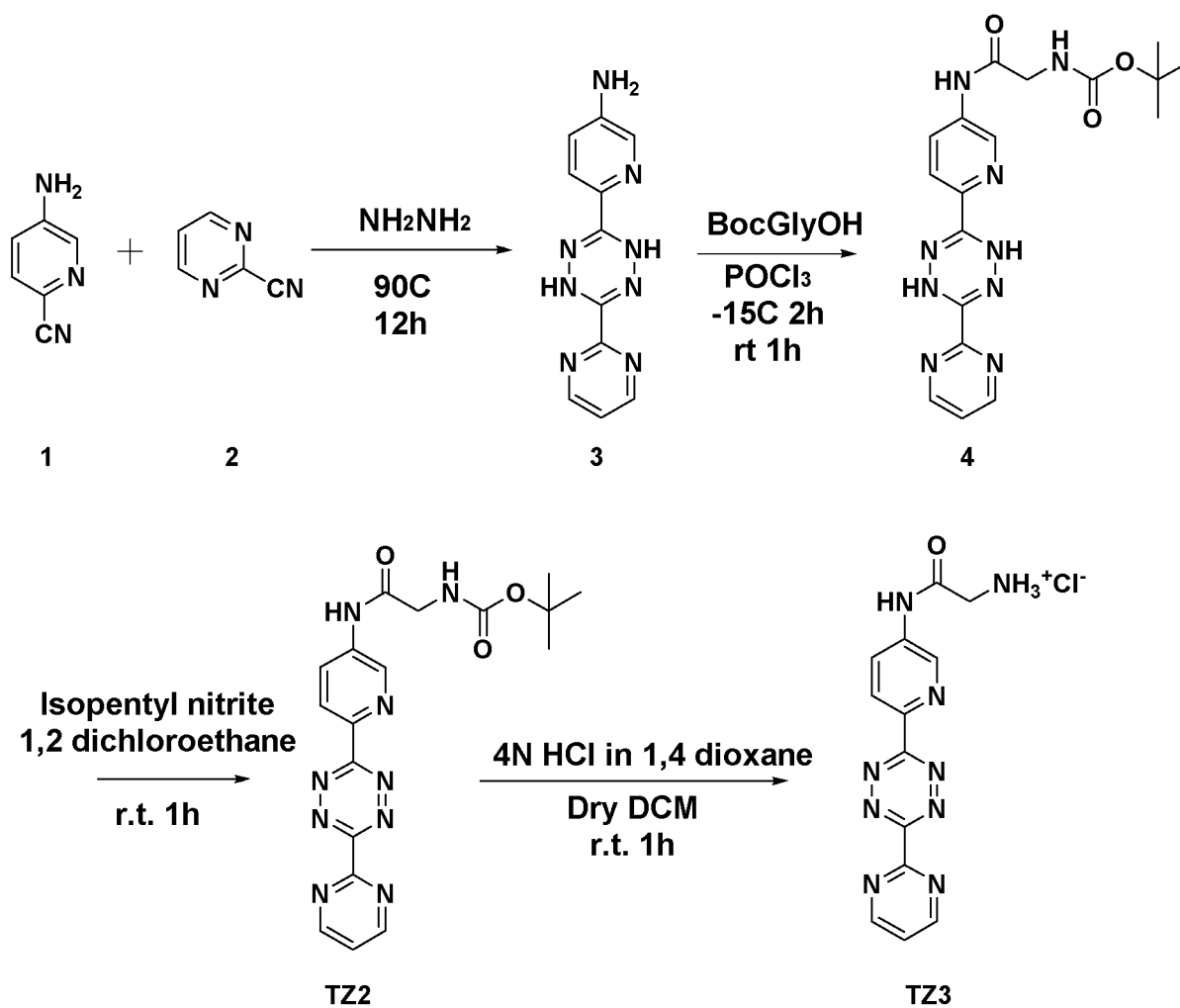
Chemical shifts are reported on the δ scale in ppm and are referenced to residual non-deuterated solvent resonances. Normal phase purifications by column chromatography were carried out on silica gel 60 (230-400 mesh). Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on an HP1100 system equipped with a Discovery C18 reverse-phase column (5 cm x 4.6 mm, 5 μ m) with a flow rate of 1 mL/min and eluting with H₂O/CH₃CN/HCOOH (95/5/0.1) to H₂O/CH₃CN/HCOOH (5/95/0.1), over 6 min, holding at 95% ACN for 2 min, with detection at 254, 500 and 650 nm and by evaporative light scattering. Electrospray ionization mass spectrometry (ESI-MS) analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) in an ESI mode. MALDI spectra were acquired on a Bruker Ultraflex extreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in H₂O/CH₃CN/TFA (50/50/0.1). Florescence monitoring experiments were done on BioTek SIAFRT.

Synthesis of Tetrazines

Tetrazines TZ1 to TZ6 (shown below) were synthesized to perform de-caging reactions. These tetrazines were used in further reactions.



Synthesis of TZ2 and TZ3



Compound 3 was synthesised according to the literature procedure.⁹

Synthesis of compound 4

5 Compound 4 was synthesized from a modified procedure.⁹ To a cold solution (-15 °C) of N-(*tert*-butoxycarbonyl)glycine (530 mg, 2.28 mmol) and 3 (401 mg, 2.08 mmol) in pyridine (11 mL), phosphoryl chloride (218 µL, 2.28 mmol) was added drop wise. The mixture was stirred for 2 hours at -15 °C and then allowed to warm to room temperature and was stirred for an additional hour. The reaction mixture was quenched with water (1mL). The solvents were
10 evaporated under reduced pressure. The crude solid was dissolved in methanol, concentrated onto silica gel and was purified by chromatography on SiO₂ (0% to 3% methanol in dichloromethane) as an orange solid (588mg, 61%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.42 (s, 1H), 9.05 (s, 1H), 8.94 (d, 2H), 8.89 (s, 1H), 8.83 (d, 1H), 8.17 (dd, 1H), 7.96 (d, 1H), 7.63 (t, 1H), 7.13 (t, 1H), 3.79 (d, 2H), 1.41 (s, 9H). ppm; ¹³C NMR (500 MHz, *d*₆- DMSO): δ 169.14,
15 157.66, 155.98, 155.91, 145.64, 145.55, 141.40, 138.95, 136.98, 126.77, 122.08, 121.49, 78.14, 43.82, 27.34 ppm; ESI-MS (*m/z*): [M+H]⁺ 412.0

Synthesis of TZ2

TZ2 was synthesised from a modified procedure.⁹ To a stirred solution of compound 4 (600
20 mg, 1.46 mmol) in 1, 2-Dichloroethane (35 ml) Isopentyl nitrite (0.39 ml, 2.9 mmol) was added dropwise at room temperature. The reaction was completed in 2 hours and the solvents were evaporated to afford TZ2 as a pink solid (525 mg, 88%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.64 (s, 1H), 9.21 (d, *J* = 4.9 Hz, 2H), 9.08 (d, *J* = 2.5 Hz, 1H), 8.68 (dd, *J* = 8.6, 0.7 Hz, 1H), 8.45 (dd, *J* = 8.7, 2.5 Hz, 1H), 7.85 (t, *J* = 4.9 Hz, 1H), 7.18 (t, *J* = 6.1 Hz,
25 1H), 3.85 (d, *J* = 6.1 Hz, 2H), 1.43 (s, 9H) ¹³C NMR (126 MHz, DMSO-*d*₆) δ 169.55 , 162.78 , 162.69 , 159.10 , 158.49 , 155.96 , 143.81 , 141.35 , 138.34 , 126.24 , 125.32 , 122.96 , 78.19 , 43.93, 28.19 ESI-MS (*m/z*): [M+H]⁺ found 410.2

TZ3 was synthesized from literature procedure.⁹

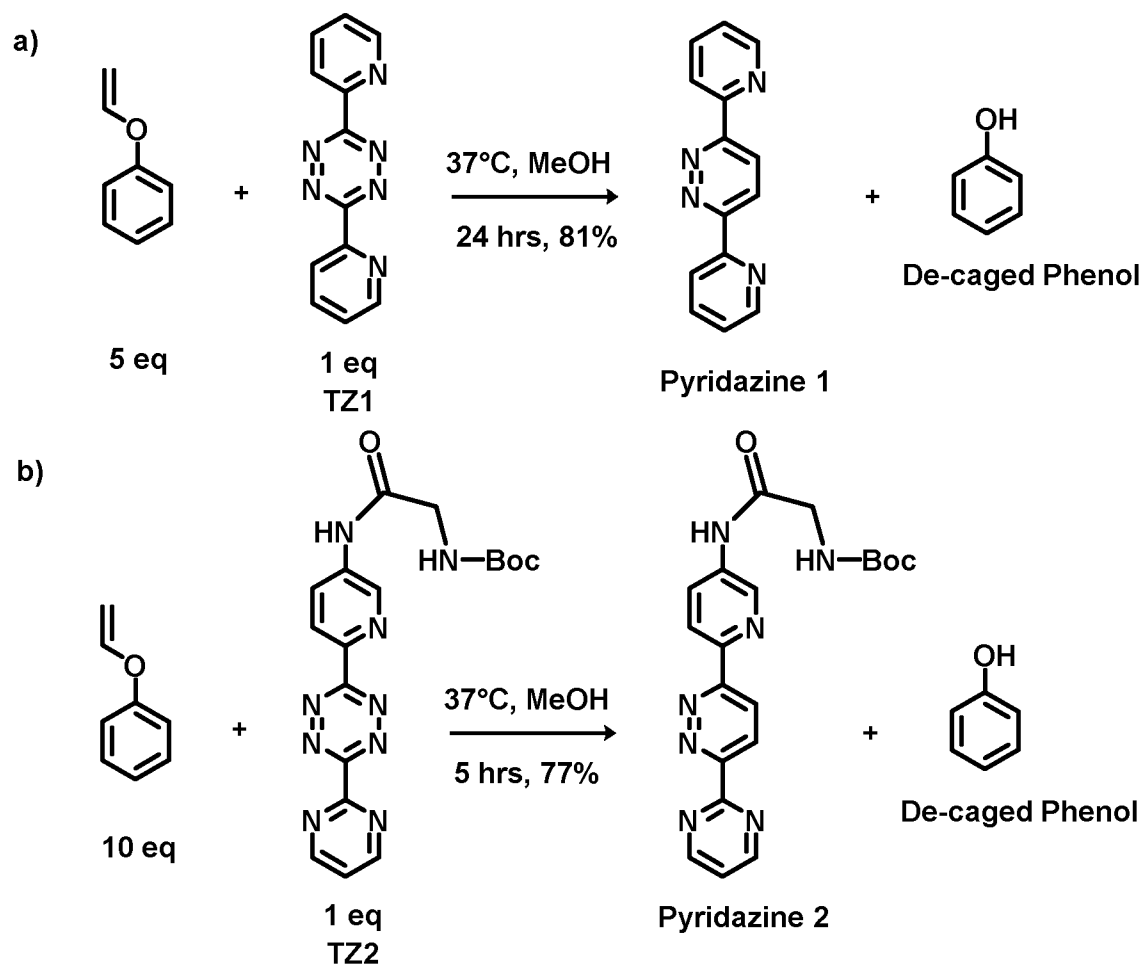
TZ5 was synthesized from literature procedure.¹⁰

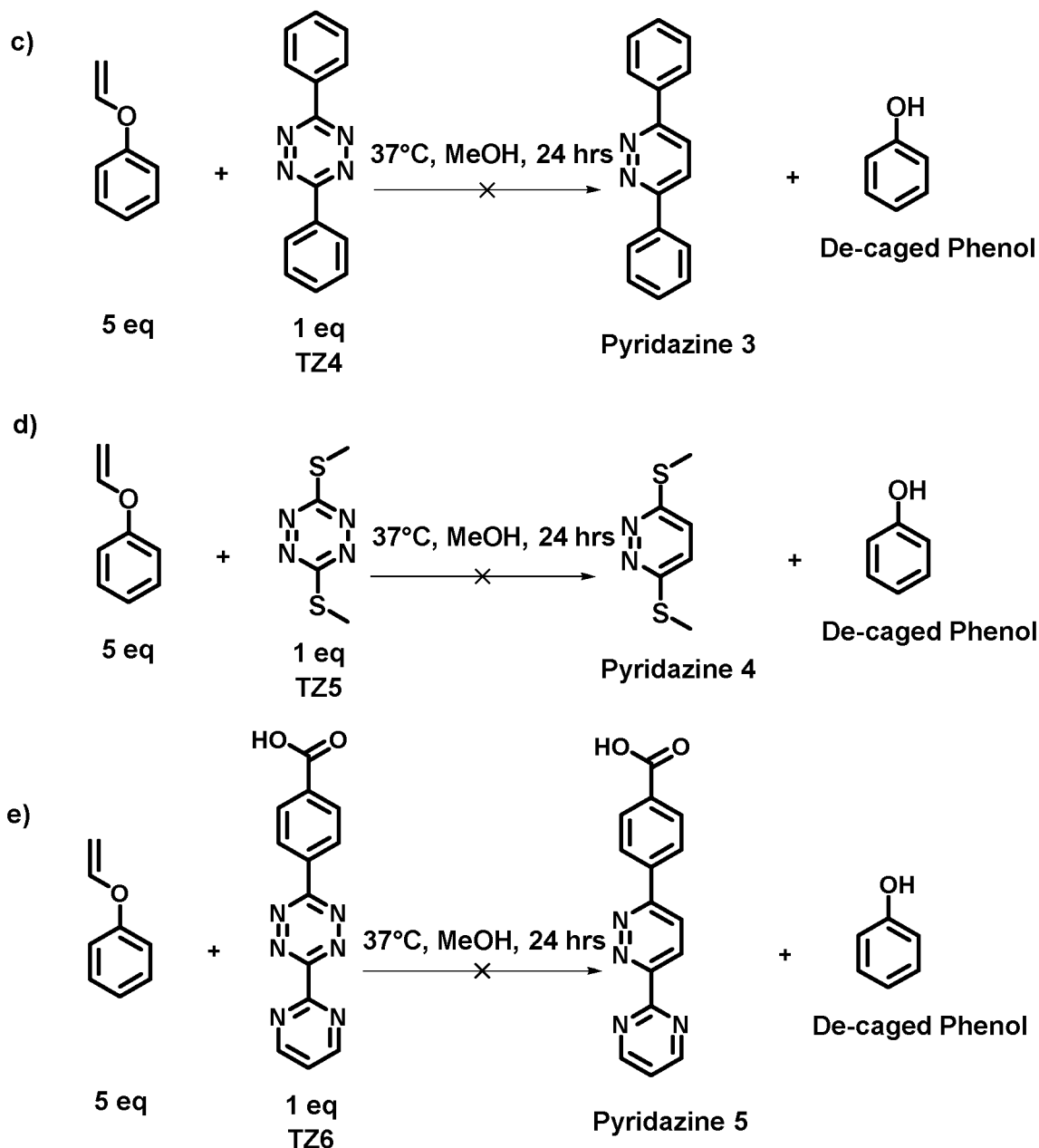
30 TZ6 was synthesized from literature procedure.¹¹

Screening of various tetrazines for De-caging of vinyl ether

Novel de-caging of vinyl ether was performed to release free hydroxyl groups. Phenylvinylether was used as model compound. Various tetrazines were used to de-cage the
35 phenylvinylether (Scheme 2a-e). The de-caging was successful with three tetrazines with different percentage of de-caging. TZ1 de-caged phenylvinylether with 81% yield and TZ3 de-caged phenylvinylether with 77% yield. Further, TZ6 mediated de-caging was monitored by

^1H NMR. TZ6 only de-caged 19% of the vinyl ether (Figure 2). All the reactions were performed at physiological temperature. TZ1, TZ2 and TZ3 were considered as ideal candidates for the novel de-caging of various caged fluorophores.





Scheme 8: Use of tetrazines TZ1-TZ6 (a-e)) to “de-cage” phenol

5

General procedure for De-caging of Phenylvinylether

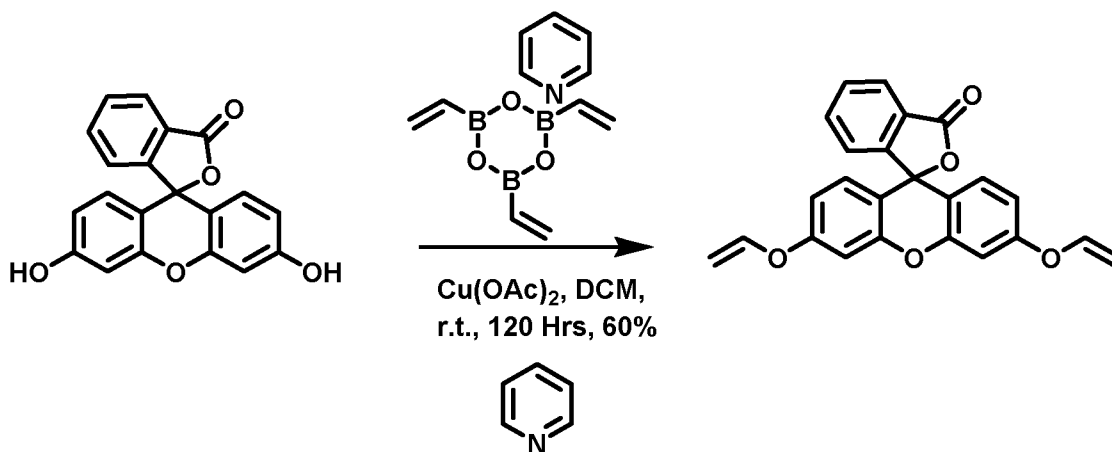
Phenylvinylether and tetrazine are mixed in MeOH. Solution was heated to 37 °C and stirred till the reaction was complete. Reaction was monitored by TLC. Further column chromatography was performed to yield pyridazine 1 (4 mg, 81%) and pyridazine 2 (19 mg, 77%).

10

Pyridazine 1 ^1H NMR (500 MHz, Chloroform-*d*) δ 8.82 – 8.74 (m, 4H), 8.72 (s, 2H), 7.94 (dt, 2H), 7.44 (m, 2H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 158.22, 153.50, 149.50, 137.21, 125.17, 124.78, 121.71. ESI-MS (*m/z*): $[\text{M}+\text{H}]^+$ found 235.2.

- 5 **Pyridazine 2** ^1H NMR (601 MHz, Chloroform-*d*) δ 9.01 (d, 2H), 8.91 – 8.73 (m, 3H), 8.68 (p, 2H), 8.23 (dd, 1H), 7.44 (t, 1H), 5.44 (t, 1H), 4.03 (d, 2H), 1.52 (s, 9H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 168.54, 162.15, 158.35, 157.86, 156.84, 148.90, 147.93, 140.82, 135.79, 127.58, 127.10, 124.47, 122.49, 121.10, 81.21, 45.91, 28.31. ESI-MS (*m/z*): $[\text{M}+\text{H}]^+$ 408.2.

10 **Synthesis of VinylFluorescein¹²**



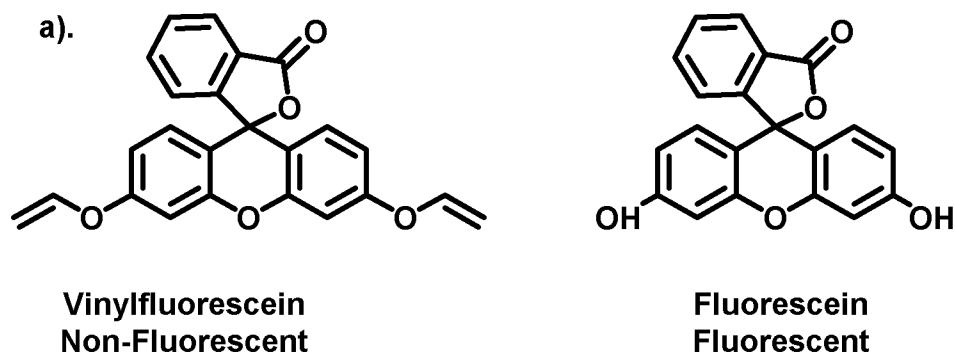
Scheme 9: Synthesis of vinyl fluorescein

- 15 $\text{Cu}(\text{OAc})_2$ (0.15 g, 0.83 mmol) was stirred at room temperature in dry CH_2Cl_2 (10 mL) for 10 min. Fluorescein (0.1 g, 0.3 mmol), Vinylboronic anhydride pyridine complex (0.13 g, 0.55 mmol), and pyridine (0.66 mL, 8.23 mmol) were added, and the reaction was stirred at room temperature for 120 h. The solvents were evaporated and column chromatography was performed using DCM:MeOH (98:2) as eluent yielding the product as a light pink solid (0.57 g, 98%). ^1H NMR (500 MHz, DMSO-*d*₆) δ 8.05 (dt, 1H), 7.82 (td, 1H), 7.76 (td, 1H), 7.33 (dt, 1H), 7.10 (d, 2H), 7.00 (dd, 2H), 6.87 (dd, 2H), 6.81 (d, 2H), 4.88 (dd, 2H), 4.61 (dd, 2H). ^{13}C NMR (126 MHz, DMSO-*d*₆) δ 168.97, 158.32, 152.79, 151.96, 147.74, 136.37, 130.91, 130.10, 126.13, 125.38, 124.50, 113.75, 113.70, 104.22, 97.52, 82.03, 79.27. ESI-MS (*m/z*): $[\text{M}+\text{H}]^+$ 285.1
- 20

25 **Masking/Caging of Fluorophore**

A novel caged fluorophore (Vinylfluorescein) was synthesised. As expected the use of vinyl ether was successful in caging the fluorophore. Vinyl ether masked the fluorescence of the

Fluorescein. This was confirmed fluorescence blocking of Fluorescein ($E_x / E_m = 490/525\text{nm}$) (Figure 1a).

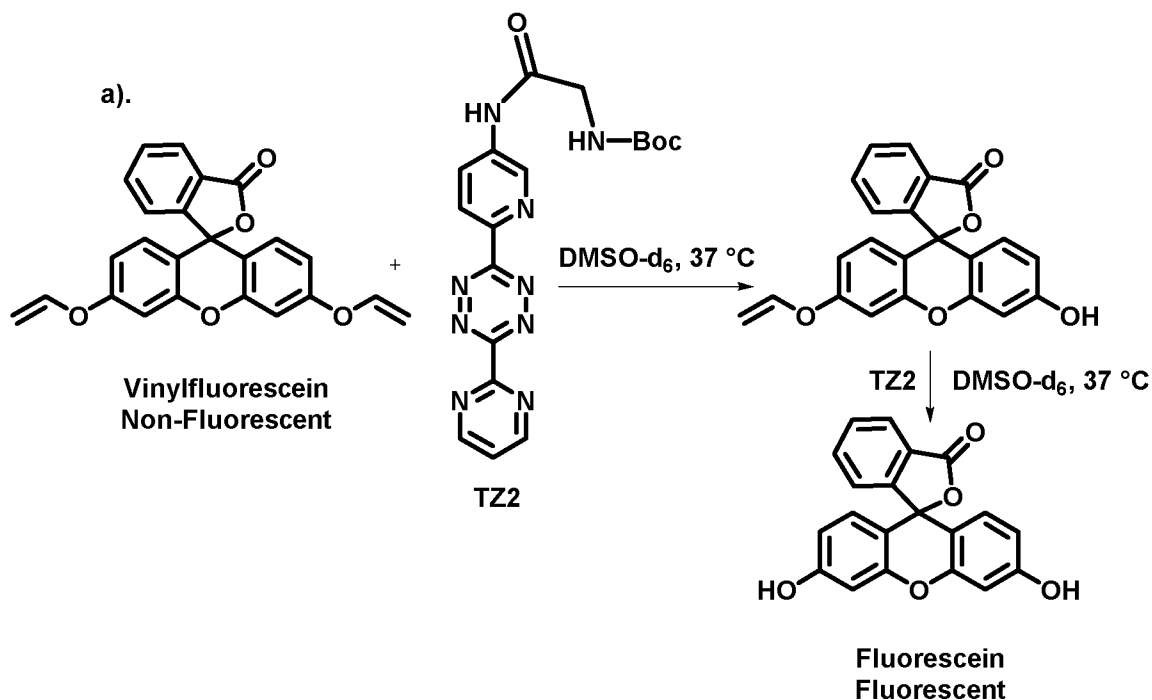


5 Scheme 10: Structures of vinylfluorescein and fluorescein

De-caging of Vinylfluorescein by TZ2 Monitored by ^1H NMR

Vinylfluorescein was de-caged by TZ2 in $\text{DMSO-}d_6$ at 37°C . 100% of vinylfluorescein reacted with TZ1 after 44 hrs giving 97% of de-caged fluorescein with 3% of mono-vinylfluorescein.

10 The reaction was monitored by ^1H NMR at intervals of 1 hr (Figure 16). This confirms the de-caging of vinyl ether by tetrazine.

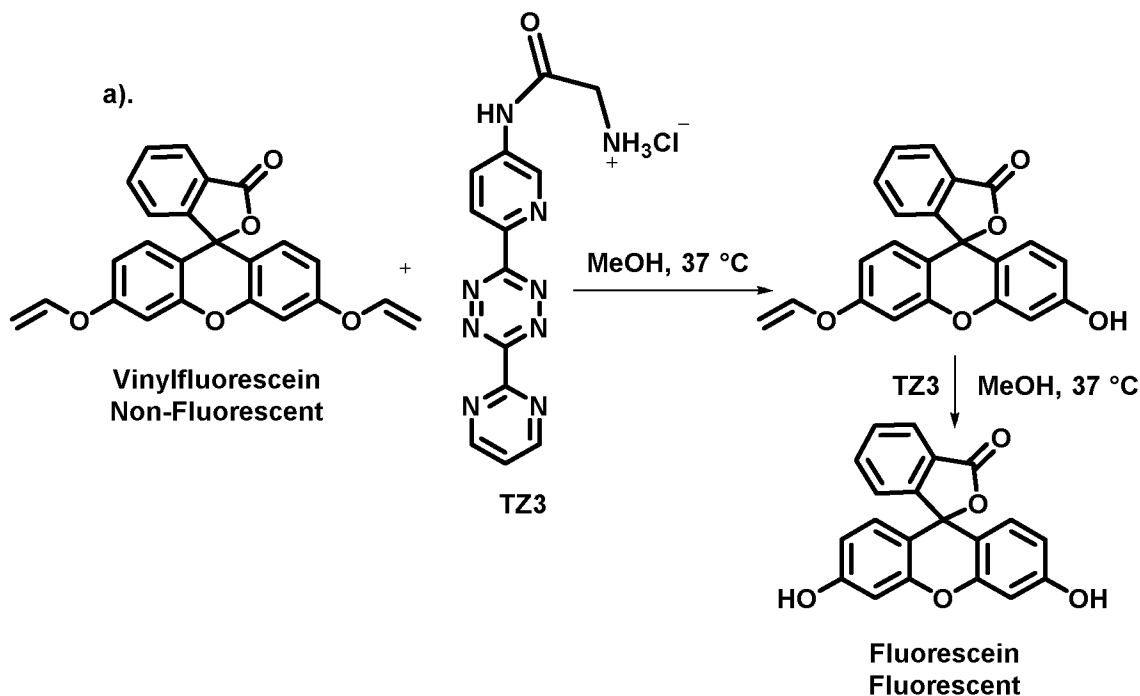


Scheme 11: Reaction between vinylfluorescein (1 eq, 2mg) and TZ2 (10 eq, 16.5 mg) in 0.6 ml in $\text{DMSO-}d_6$ at 37°C .

15

3.8 De-caging of Vinylfluorescein by TZ3 Monitored by HPLC

TZ3 was used for further experiments as its solubility in aqueous media was found to be 1mM. Vinylfluorescein was de-caged by TZ3 in MeOH at 37° C. The reaction was monitored by HPLC (Figure 4). After 24 hrs 7.48%, 20.16% and 71.89% of vinylfluorescein, mono-vinylfluorescein and fluorescein was found (Figure 5 and Table 1). This further confirms the de-caging of vinyl ether by tetrazine.



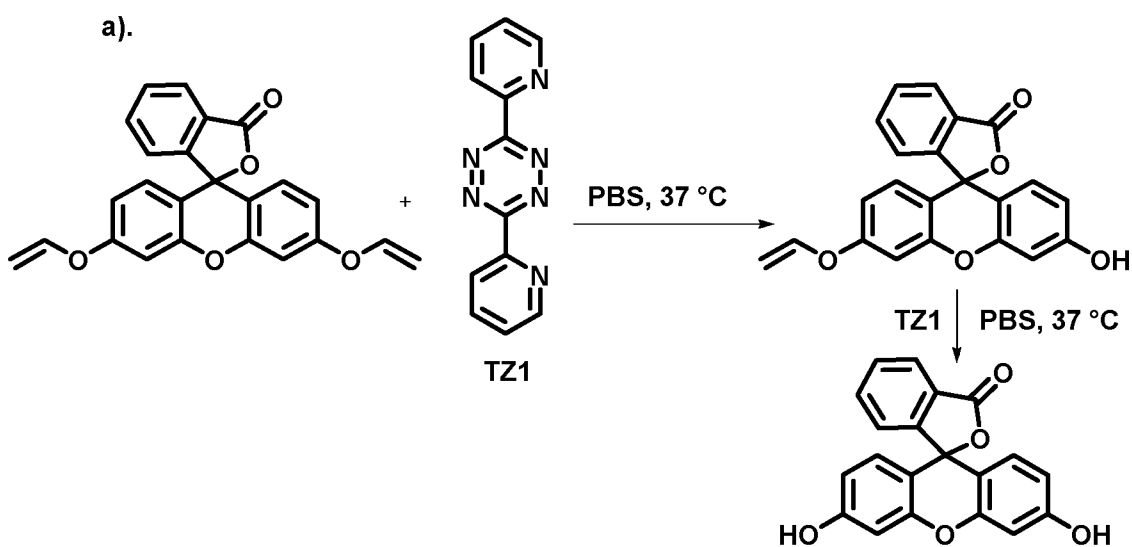
Scheme 12: Reaction between vinylfluorescein (125 μ M) and TZ3 (1mM) in MeOH at 37°C.

Compound	Ret. Time (min)	Area %
Fluorescein	6.158	71.89
Mono- vinylfluorescein	7.158	20.61
Vinylfluorescein	7.802	7.48

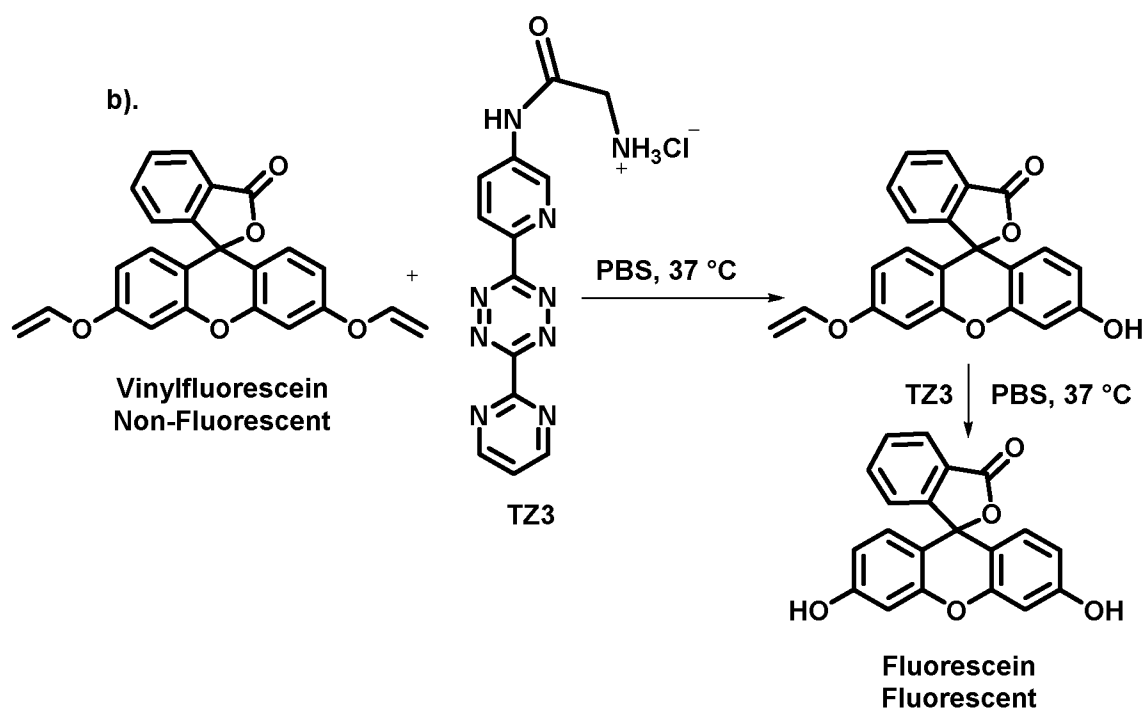
Table 1 Peak Area and retention time after 24 hrs of fluorescein, mono-vinylfluorescein and vinylfluorescein from HPLC chromatogram (Figure 5).

De-caging of Vinylfluorescein by TZ1 and TZ3 Monitored by Fluorescence

The de-caging of fluorescein was monitored by increase in fluorescence in comparison to vinylfluorescein. The novel de-caging reaction was performed at 37 °C and in phosphate saline buffer (PBS). TZ1 (200μM) reacted with vinylfluorescein (35μM) to give 20 fold increase in fluorescence after 40 hrs (Scheme 13a and Figures 6 and 7). Further, TZ3 (500μM) reacted with vinylfluorescein (35μM) to give 123 fold increase in fluorescence after 24 hrs (Scheme 13b and Figures 8 and 9). In all the cases vinylfluorescein was found to be stable, showing stability of vinyl ether in PBS and at physiological temperature for 40 hours. This technique confirms the novel de-caging of vinyl ether in real time.



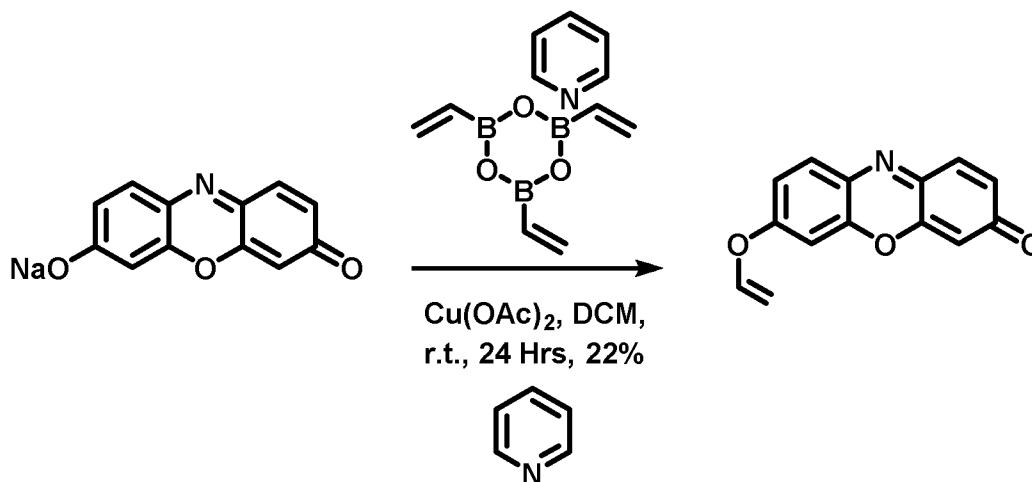
10



Scheme 13: Reaction between a) vinylfluorescein (35 μM) and TZ3 (500 μM) in PBS at 37°C and b) between vinylfluorescein (35 μM) and TZ1 (200 μM) in PBS at 37 °C.

Synthesis of Fluorophore (Vinylresorufin)¹²

5



Scheme 14: Synthesis of Vinylresorufin

Cu(OAc)₂ (0.07 g, 0.42 mmol) was stirred at room temperature in dry CH₂Cl₂ (5 mL) for 10 min. Resorufin sodium salt (0.1 g, 0.42 mmol), Vinylboronic anhydride pyridine complex (0.06 g, 0.28 mmol), and pyridine (0.33 mL, 4.2 mmol) were added, and the reaction was stirred at room temperature for 24 h. The solvents were evaporated and column chromatography was performed using DCM:MeOH (98:2) as eluent yielding the product as a light pink solid (0.22 g, 22%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.78 (d, 1H), 7.45 (d, 1H), 7.05 (dd, 1H), 6.96 (d, 1H), 6.87 (dd, 1H), 6.72 (dd, 1H), 6.35 (d, 1H), 5.04 (dd, 1H), 4.73 (dd, 1H). ¹³C NMR (126 MHz, Chloroform-*d*) δ 186.30, 160.23, 149.60, 146.71, 145.96, 145.34, 134.77, 134.63, 131.79, 129.51, 114.60, 107.04, 103.16, 99.22. ESI-MS (*m/z*): [M+H]⁺ 240.1

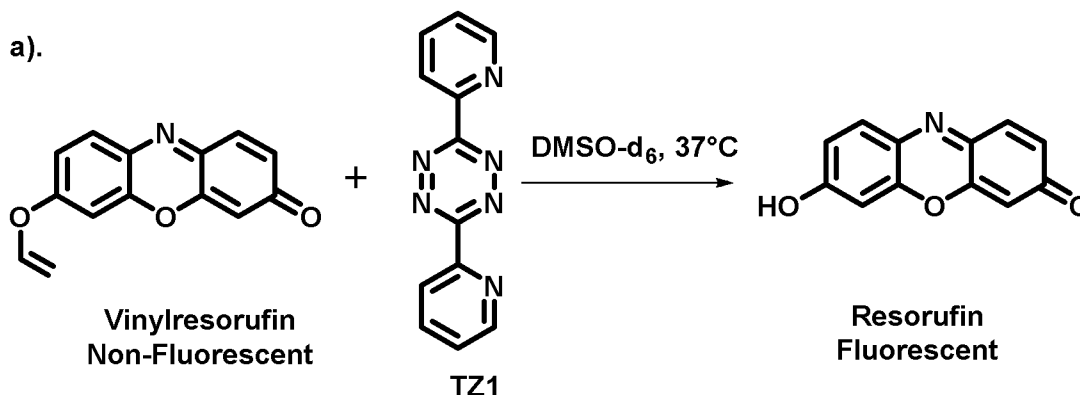
Masking of Resorufin

Novel fluorophore (Vinylresorufin) was synthesised (Figure 10a). As expected the use of vinyl ether was successful. Vinyl ether masked the fluorescence of the resorufin. This was confirmed by the blocking of Fluorescence intensity of Resorufin (*E*_x / *E*_m = 570 / 585 nm) (Figure 10b).

De-caging of Vinylresorufin via Tetrazines

- De-caging of vinylresorufin by TZ1 monitored by ¹H NMR

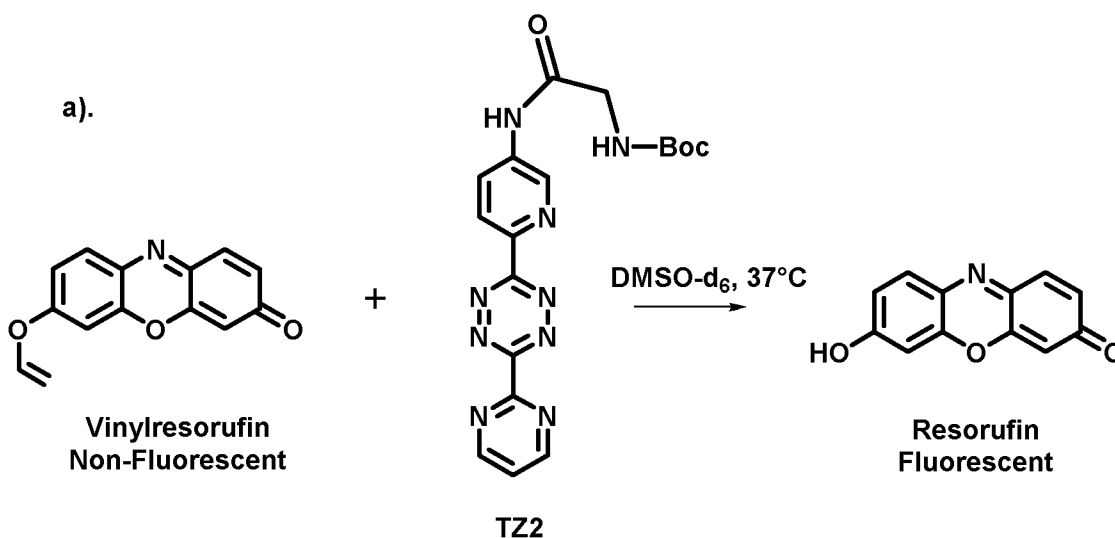
Vinylresorufin was de-caged by TZ1 in DMSO- d_6 at 37° C. Only 73% of vinylresorufin was remaining after 17 hrs giving 27% of de-caged resorufin. The reaction was monitored by ^1H NMR at intervals of 1 hr (Figure 11).



- 5 **Scheme 15: Reaction between vinylfluorescein (1 eq, 2mg) and TZ2 (5 eq, 14.5 mg) in 0.6 ml in DMSO- d_6 at 37°C.**

- **De-caging of vinylresorufin by TZ2 monitored by ^1H NMR**

- 10 Vinylresorufin was de-caged by TZ2 in DMSO- d_6 at 37° C (Scheme 15). Only 30% of vinylresorufin was remaining after 17 hrs giving 70% of de-caged resorufin (Figure 12). The reaction was monitored by ^1H NMR at intervals of 1 hr (Figure 13).



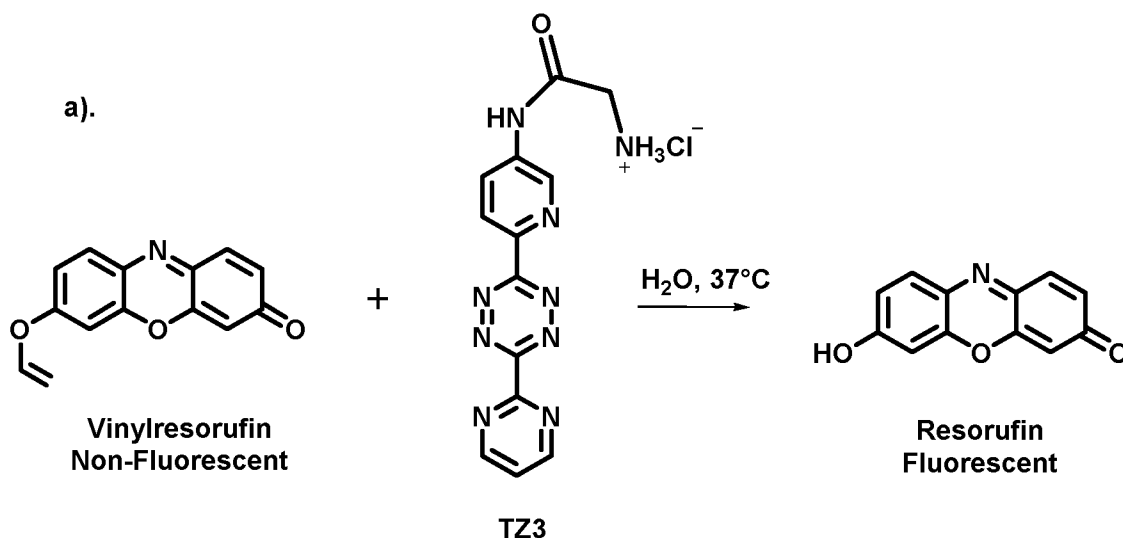
Scheme 16: Reaction between Vinylresorufin (1 eq, 2mg) and TZ2 (5 eq, 14.5 mg) in 0.6 ml in DMSO- d_6 at 37°C.

15

De-caging of Vinylresorufin by TZ3 Monitored by HPLC

TZ3 was used for further experiments as its solubility in aqueous media was found to be 1mM. Vinylresorufin was de-caged by TZ3 in water at 37° C (Scheme 16). The reaction was monitored by HPLC (Figure 14). After 24 hrs 3.7%, 51.9% and 44.3% of TZ3, vinylresorufin

and resorufin was found (Figure 15 and Table 2). This further confirms the de-caging of vinyl ether by tetrazine.



5 **Scheme 17: Reaction between vinylresorufin (125 μM) and TZ3 (1mM) in water at 37 $^\circ\text{C}$.**

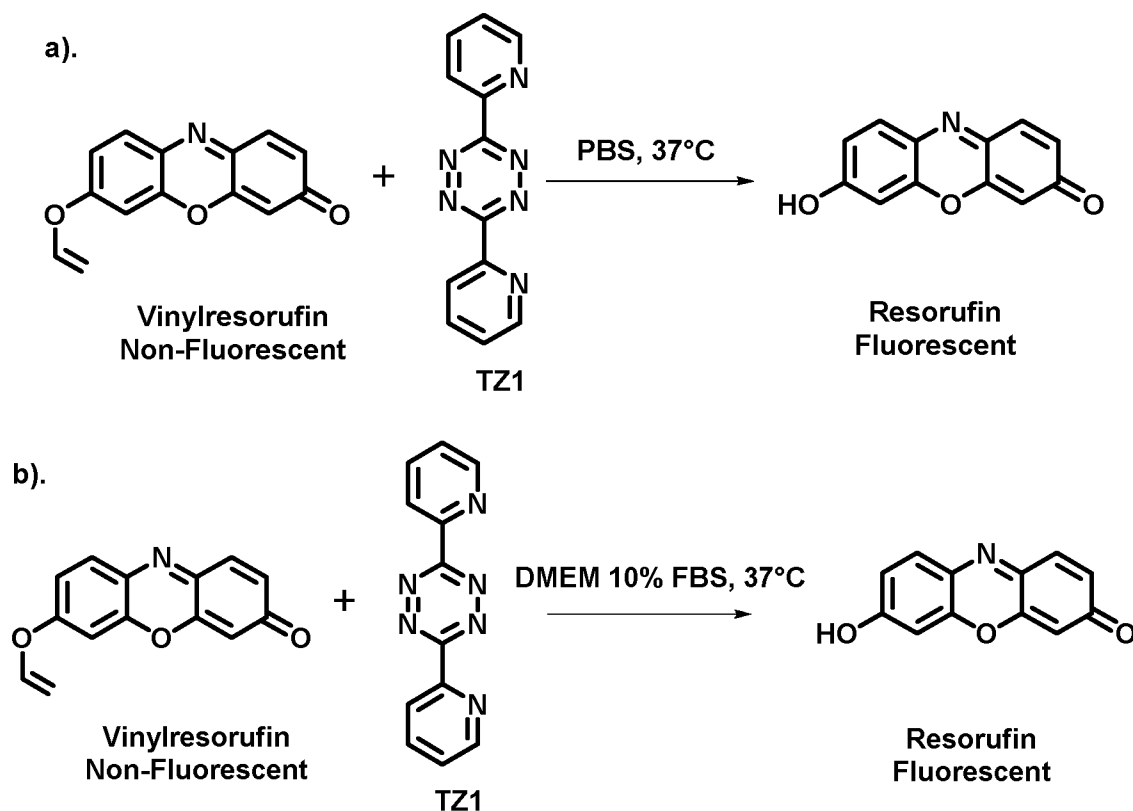
Compound	Ret. Time (min)	Area %
Tetrazine (TZ3)	2.862	3.732
Resorufin	5.135	51.959
Vinylresorufin	7.468	44.309

Table 2 Peak Area and retention time after 24 hrs of tetrazine TZ3, resorufin, and vinylresorufin from HPLC chromatogram (Figure 15).

20 **De-caging of Vinylresorufin by TZ1 and TZ3 Monitored by Fluorescence**

The de-caging of resorufin was monitored by increase in fluorescence in comparison to vinylresorufin. The novel de-caging reaction was performed at 37 $^\circ\text{C}$ and in phosphate saline buffer (PBS) or DMEM with 10% FBS (Dulbecco's Modified Eagle Medium with 10% Fetal bovine serum). TZ1 (200 μM) reacted with vinylfluorescein (10 μM) in PBS to give 32.6 fold increase in fluorescence after 40 hrs (Scheme 18, Figures 16 and 17). Further, TZ3 (500 μM) reacted with vinylresorufin (100 μM) to give 10.8 fold increase in fluorescence after 5 hrs

(Scheme 13, Figures 18 and 19). In all the cases vinylresorufin was found to be stable, showing stability of vinyl ether in PBS and DMEM with 10% FBS at physiological temperature. This technique confirms the novel de-caging of vinyl ether in real time.



Scheme 18: Reaction between vinylresorufin (100 μ M) and TZ3 (2 mM) in DMEM with 10% FBS at 37°C.

10 Toxicity of Caged Fluorophore and Tetrazine

MTT assay which determines the cell viability was performed to determine the toxicity of the vinylfluorescein, fluorescein, vinylresorufin, resorufin, TZ1 and TZ3. The various concentrations were tested to find the optimum safe concentration to be used in further cell assays. TZ1 and TZ3 showed no toxicity in comparison to control cells at concentration of 10 μ M and 100 μ M respectively. Vinyl ether caged fluorescein showed no toxicity even at concentrations of 200 μ M (Figure 20 and 21).

Synthesis of Caged Drug

Divinyl-5, Fluorouracil was synthesised as a masked drug of 5-Fluorouracil. 5-Fluorouracil kills the cells through irreversible inhibition of thymidylate synthase.

Synthesis of Divinyl-5-Fluorouracil

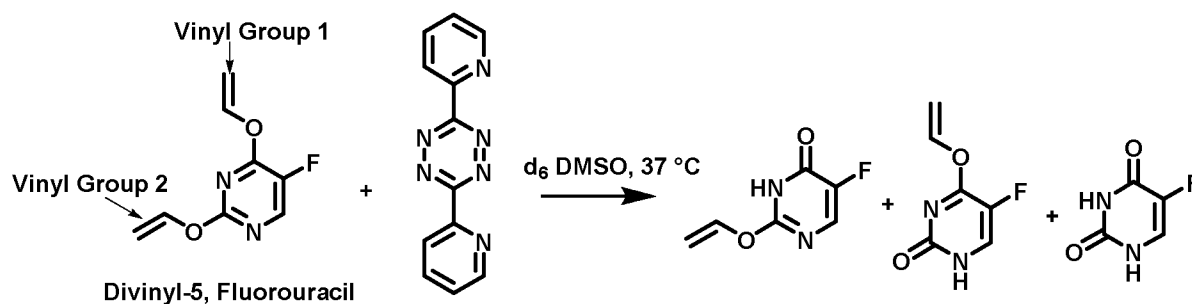
A pro-drug form of 5-fluorouracil, the monovinyl-form, was made using the following synthesis.

- 5 In a flask 5-Fluorouracil (25 mg, 0.2 mmol, 1 equiv.), 2,4,6-Trivinylcyclotriphosphazene-pyridine (48 mg, 0.2 mmol, 1 equiv.), caesium carbonate (65 mg, 0.2 mmol, 1 equiv.) and copper (II) acetate (36 mg, 0.2 mmol, 1 equiv.) were added. The compounds were dissolved in anhydrous acetonitrile (2 mL). The solution has been stirred for 24 h at room temperature.
- 10 After 3 days the reaction was quenched with aq. Ammonia acetate buffer (3 M, 10 mL) and the solution was stirred for additional 30 min. After that the solution was washed with ethyl acetate (15 mL) three times. The collected organic layers were washed with brine (15 mL). After drying with Na₂SO₄, filtration and evaporation of the solvent the crude product was purified with silica column (hexane/ethyl acetate, 6/4). The product obtained as a white solid
- 15 (12.6 mg, 35 %).

¹H-NMR (500 MHz, CDCl₃): δ 5.01 (m, 1H); 5.24 (m, 1H); 5.41 (m, 1H); 5.99 (m, 1H); 6.79 (m, 1H); 7.21 (m, 1H); 7.91 (m, 1H).

20 De-caging of Divinyl-5, Fluorouracil by TZ1 monitored by ¹H NMR

TZ1 was used to de-cage the Divinyl-5, Fluorouracil. The two vinyl group (1 & 2) were de-caged 20% and 18% respectively (Figure 22).



25 Scheme 19 Structure of Divinyl-5,Fluorouracil

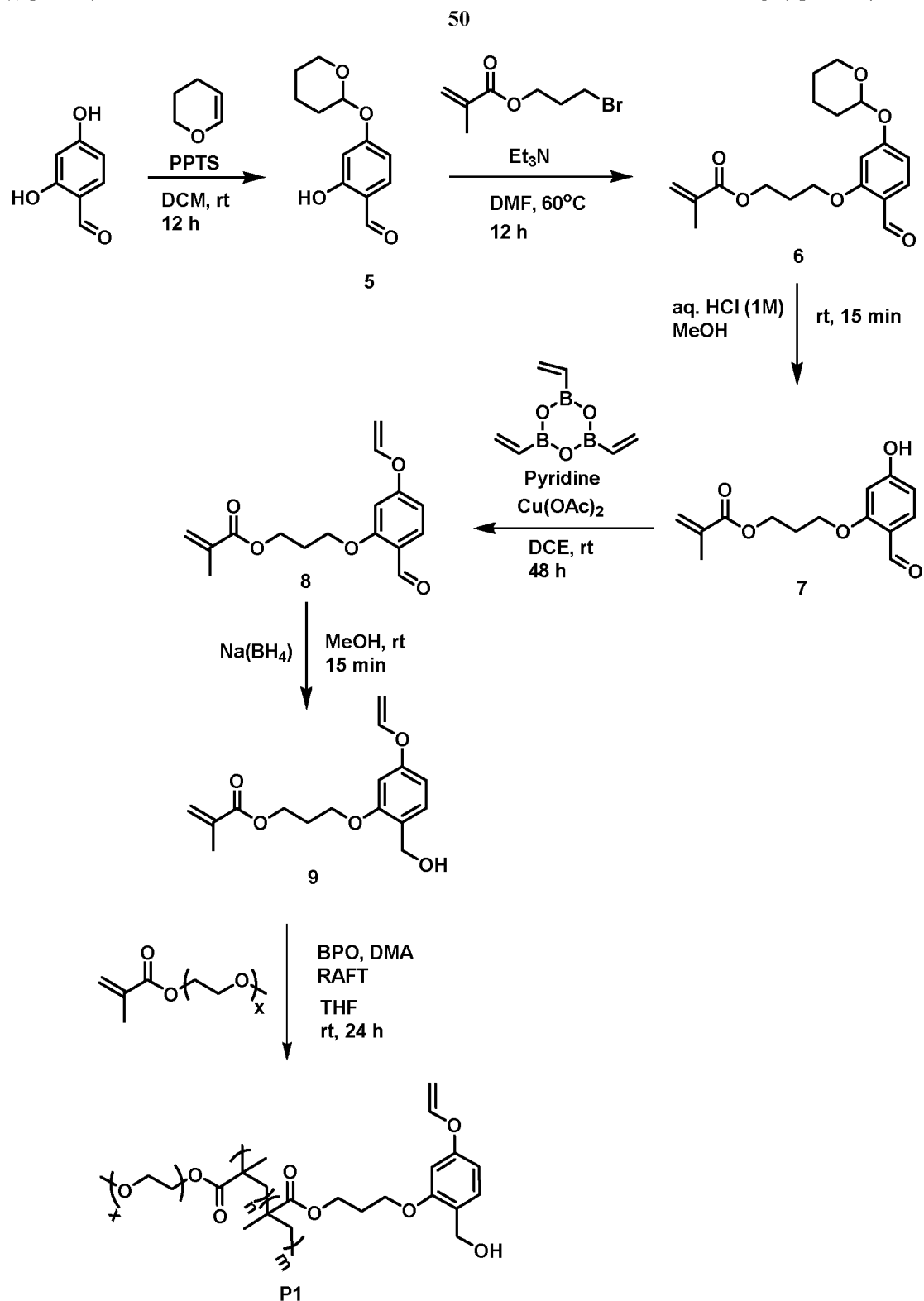
It has been found (data not shown) that the divinyl and monovinyl forms of fluorouracil are less therapeutically active than the free form.

30 Synthesis of a polymer for use as a caging group to mask active agents.

Polymers may be used as a caging group to mask active agents that comprise a polymer backbone having different properties, such as improved solubility and tissue targeting capabilities.

- 5 An example of one such polymer is synthesized polymer P1 (synthesised as shown in Scheme 20 below), which shows two features. First of all, it shows the possibility to polymerize methacrylate without affecting the vinyl ether moiety (for that reason redox-sensitive BPO is used instead of thermal AIBN). Secondly, the reaction between tetrazine and polymer P1 shows a similar reactivity compared to previous results (reaction between two small
10 molecules). Therefore, it seems that even a bulky polymer is not decreasing the reactivity between vinyl ether and tetrazine.

The reaction of P1 with tetrazine is shown in Figure 23.



Scheme 20 Synthetic pathway for polymer P1.

2-Hydroxy-4-O-tetrahydropyranylbenzaldehyde (5)

- 5 Starting material 2,4-Dihydroxybenzaldehyde (500 mg, 3.6 mmol) have been add together with 2,4-Dihydro-2H-pyran (488 mg, 5.8 mmol) and dissolved in absolute CH_2Cl_2 (40 mL). After the solids were dissolved pyridinium *p*-toluenesulfonate (910 mg, 3.6 mmol) was added.

The reaction solution was stirred at ambient temperature for 4 h. After reaction the solution was washed with sodium phosphate buffer pH 6.8 (3 x 50 mL) and brine (1 x 50 mL). The organic phase's layers were dried over anhydrous Na₂SO₄. After filtration and evaporation of the solvent the product was purified by silica gel column (v:v, Hexane:EtOAc, 7:3). The product (454 mg, 2.05 mmol) obtains as an oil in moderate yield (58 %).

¹H-NMR (500 MHz, CDCl₃): δ 1.59 – 1.92 (m, 6H); 3.65 (m, 1H); 3.81 (m, 1H); 5.52 (t, 1H); 6.64 (s, 1H); 6.69 (d, *J* = 8.6 Hz, 1H); 7.43 (d, *J* = 8.6 Hz, 1H); 9.73 (s, 1H); 11.46 (s, 1H). ¹³C-NMR (125 MHz, CDCl₃): δ 18.65, 25.18, 30.17, 62.40, 69.45, 103.89, 109.61, 115.98, 135.49, 164.39, 164.55, 149.77.

2-O-(methacrylate propyl ester)-4-O-tetrahydropyranylbenzaldehyde (6)

2-Hydroxy-4-O-tetrahydropyranylbenzaldehyde (536 mg, 2.4 mmol, 1 equiv.) and 3-bromo(methacrylate ester) (550 mg, 2.7 mmol, 1.1 equiv.) were dissolved in dry DMF (5 mL). After that Caesium Carbonate (1.02 g, 3.12 mmol, 1.3 equiv.) was added and the solution was stirred over night at 50°C.

Water (20 mL) was added and the solution was extracted with Ethyl acetate (3 x 40 mL). The collected organic layer have been washed with water (4 x 20 mL) and brine (1 x 30 mL). After drying over Na₂SO₄, filtration and evaporation the crude product was purified with silica column. The product obtain as an oil (626 mg, 1.8 mmol, 75 %).

¹H-NMR (500 MHz, CDCl₃): δ 1.59 – 1.94 (m, 6H); 2.01 (m, 3H); 2.24 (m, 2H); 3.62 (m, 1H); 3.84 (m, 1H); 4.10 (m, 2H); 4.37 (m, 2H); 5.52 (t, 1H); 5.57 (m, 1H); 6.11 (m, 1H); 6.62 (s, 1H); 6.69 (d, 1H); 7.78 (d, 1H); 9.73 (s, 1H); 10.33 (s, 1H).

2-O-(methacrylate propyl ester)-4-hydroxybenzaldehyde (7)

Starting material 6 (500 mg, 1.4 mmol, 1 equiv.) was dissolved in Methanol (10 ml). After that hydrochloric acid (1 M, 2 ml) was added and the solution was stirred for 15 min at ambient temperature.

After stirring the solution was extracted with Ethyl acetate (3 x 30 ml). The combined organic layers were washed with brine (20 ml). After drying over Na₂SO₄, filtration and evaporation of the solvent the product was purified with silica column (Hexane/Ethyl acetate, 6/4). The product obtained as a colourless oil in moderate yield (304 mg, 85 %).

¹H-NMR (500 MHz, CDCl₃): δ 1.95 (m, 3H); 2.25 (m, 2H); 3.65 (m, 1H); 4.15 (t, 2H); 4.38 (t, 2H); 5.59 (m, 1H); 6.12 (m, 1H); 6.49 (m, 1H); 6.56 (m, 1H); 7.75 (m, 1H), 8.59 (bs, 1H), 10.25 (s, 1H).

5 **2-O-(methacrylate propyl ester)-4-O-vinyletherbenzaldehyde (8)**

In a flask starting material **7** (300 mg, 1.1 mmol, 1 equiv.), 2,4,6-trivinylcyclotriboroxane-pyridine (180 mg, 0.75 mmol, 1 equiv.), caesium carbonate (243.75 mg, 0.75 mmol, 1 equiv.) and copper (II) acetate (135 mg, 0.75 mmol, 1 equiv.) were added. The compounds were dissolved in anhydrous dichloroethane (4 mL). The solution has been stirred for 24 h at room
10 temperature.

After the reaction is completed aqu. ammonia acetate buffer (3 M, 10 mL) was added and the solution was stirred for additional 30 min. After that the solution was washed with ethyl acetate (15 mL) three times. The collected organic layers were washed with brine (15 mL). After drying
15 with Na₂SO₄, filtration and evaporation of the solvent the product was purified with silica column (hexane/ethylacetate, 6/4) and obtained as a solid (166 mg, 50 %).

¹H-NMR (500 MHz, CDCl₃): δ 1.98 (m, 3H); 2.28 (m, 2H); 4.20 (t, 1H); 4.40 (t, 2H); 4.65 (m, 1H); 4.68 (m, 1H); 5.61 (m, 1H); 6.14 (m, 1H); 6.58 (m, 1H); 6.68 (m, 1H); 6.71 (m, 1H), 7.87
20 (m, 1H); 10.38 (s, 1H).

2-O-(methacrylate propyl ester)-4-O-vinyletherbenzalcohol (9)

Starting material **8** (100 mg, 0.34 mmol, 1 equiv.) was dissolved in Methanol (15 ml). Sodium borohydride (40 mg, 1 mmol, 3 equiv.) was added and the solution was stirred for 15 min. After
25 that the reaction was followed by TLC until completion. After 1 h the reaction went to full completion.

For purification the reaction solution was quenched with cooled (10 ml) water and extracted with DCM (30 ml) twice. After drying over sodium sulphate and evaporation of the solvent the
30 product obtained in quantitative yield as a white solid (97 mg, quant.).

¹H-NMR (500 MHz, MeOD): δ 1.95 (m, 3H); 2.19 (m, 2H); 4.11 (t, 1H); 4.37 (t, 2H); 4.42 (m, 1H); 4.61 (s, 2H); 4.70 (m, 1H), 5.63 (m, 1H); 6.12 (m, 1H); 6.60 (m, 1H); 6.64 (m, 1H); 6.75 (m, 1H), 7.31 (m, 1H).

Poly(PEG-co-*p*-vinyletherbenzalcohol) P1

PEG Mn=480 (400 mg, 0.83 mmol) and the monomer **9** (71 mg, 0.24 mmol) were given together and dissolved in THF (0.6 mL). The solution was degassed via nitrogen flow. DMA (6.9 μ L, 1 equiv.), BPO (13 mg, 1equiv.) and 2-Cyano-2-propyl dodecyl trithiocarbonate (19 mg, 1 equiv.) was added. The solution was degassed again. After degassing the solution was stirred at room temperature for 24 h.

For purification the solvent was evaporated and the oil was dissolved in water. The clear solution was dialyzed for 48 h. Water was removed by freeze drying.

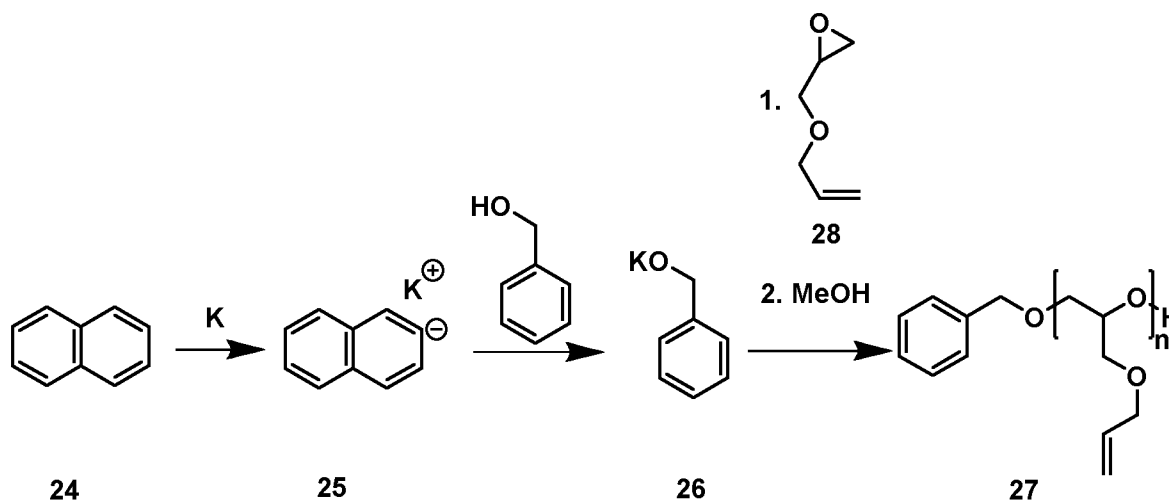
GPC (DMF): M_n 6020 g mol⁻¹, PDI 1.32.

¹H-NMR (500 MHz, d⁴-MeOD): 0.83-1.48 (MA-backbone, 80H); 1.53-1.78 (raft-initiator, 20H); 3.52-3.78 (PEG, 460H); 4.01-4.31 (-O-(CH₂)₃-O-, 51H); 4.18 (H-CH=CH-O, 4H); 4.51-4.62 (H-CH=CH-O, 4H), 4.51-4.62 (Ar-CH₂-OH, 8H), 6.57-6.69 (Ar-H, 8H); 6.71-6.83 (H-CH=CH-O, 4H), 7.18-7.32 (Ar-H, 4H).

Release of Encapsulated Active Agents Triggered by Tetrazine

Caging moieties comprising polymers may allow triggered release of active agents in situ.

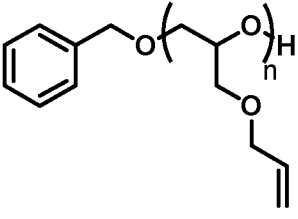
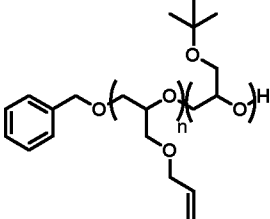
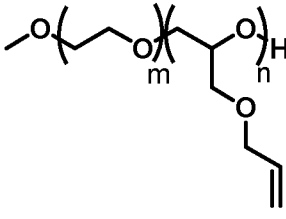
An example is the use of Poly (allyl glycidyl ether) (PAGE) polymers that react with tetrazine via the inverse Diels Alder reaction. PAGE is synthesized by an anionic ring opening polymerization of the glycidyl group (**28**) to form a polymer comprising multiple allyl ether moieties (Scheme 21).



Scheme 21 Anionic ring opening polymerisation of allyl glycidyl ether initiated by naphthalene anion.

Further examples of polymers comprising multiple allyl ether groups are given in Table 3 below.

5 Table 3 Structures and analytical data of synthesised PAGE.

			
Polymer	Poly(AGE)-homo-polymer	Random Poly(AGE)-co-(tBuGE)	Block PEG-co-PAGE
PDI	1.05 - 1.30	1.10	1.03
Mn (g/mol)	500 (P2) 9,000 (P3) 40,000 (P4)	8,000 (P5)	9,000 (P6)

This experiment illustrates the strong dependence of the polymer-post-modification by the size of the polymer. Smaller polymers react much faster than polymers with higher molecular weight. Overall the experiments show that for medium sized polymers around 9 kDa full conversion can be achieved after 16 h at moderate temperature.

Reaction of Polymer Nanoparticles with Tetrazine

Nanoparticle were prepared by dissolving block PEG-co-PAGE (P6) in DMF and followed by precipitation in water. (Figure 27) The hydrophobic part of the polymer becomes more hydrophilic after reacting with tetrazine

Both the DLS experiments as well as the TEM images show that the size of the nanoparticle is significantly decreased after adding tetrazine (Figure 27).

Release of active agents from Nanoparticles by tetrazine activation

The release profile (Figure 28) confirms the trigger to release the cargo encapsulated by PAGE nanoparticles with tetrazine.

Polymer conjugated active agents

To demonstrate the potential of external small-molecule controlled cargo release, the activated linker 9 in Scheme 20 above was conjugated to the anti-cancer agent Doxorubicin (Figure 29a). The Doxorubicin monomer showed >90% conversion to the free drug after 5 day incubation with tetrazine TZ1 (Figure 29b). The methacrylate moiety of the Doxorubicin monomer was polymerized with the RAFT (Reversible Addition-Fragmentation chain Transfer) reagent PEG CTA (Poly(ethylene glycol) 4-cyano-4-(phenylcarbonothioylthio)pentanoate, M_n 10,000 g mol⁻¹) using APS/TMEDA as the redox initiator to give the amphiphilic PEG-*b*-Dox co-polymer **15** of Figure 29a (13,000 g mol⁻¹). These mild reaction conditions were required as thermally or UV initiated polymerizations led to co-reaction of the vinyl ether groups. Once placed in water the PEG-*b*-Dox co-polymer **15** formed nanoparticles with a diameter of 35 nm (Figure 29c), with the hydrophobic core of each particle, consisting of the four Doxorubicin units (determined by ¹H NMR), linked to the methacrylate backbone surrounded by a hydrophilic PEG shell.

Tetrazine mediated controlled drug release was explored using HEK273T cells. The PEG-*b*-Dox nanoparticles (loading equivalent to 8 μM of Dox) showed no cytotoxicity (MTT assay) after 48 h, whereas 1 μM “free” Doxorubicin resulted in complete cell death. Tetrazine TZ1 showed no toxicity at a concentration of 35 μM. When the cells were treated with 1 μM PEG-*b*-Dox nanoparticles (equivalent to 4 μM of Dox), the addition of tetrazine TZ1 (35 μM) triggered cytotoxicity with 73 % cell death after 48 h incubation (Figure 29). This indicates that the nanoparticles underwent efficient tetrazine triggering, even in a complex cellular environment, leading to a controlled switch-on of cytotoxicity.

The above demonstrates the practicality and application of a tetrazine-activated self-immolative linker, which allows the controlled release of fluorophores and drugs within a complex biological milieu. Nanoparticles containing multiple covalently attached Doxorubicins (attached via a 4-hydroxymethyl phenyl vinyl ether linker) demonstrated efficient tetrazine mediated switch-on of cytotoxicity via a 1,6-elimination driven release. In the absence of a tetrazine stimulus, the PEG- *b*-Dox nanoparticles display low cytotoxicity and inherently have EPR targeting abilities.

Polymer encapsulated active agent

Poly(allyl glycidyl ethers) (PAGE) have been explored as a chemically flexible alternative to PEG, stemming from the pendant allyl groups which are amenable to a range of modifications. In this study, a diblock copolymer, poly(ethylene glycol)-*b*-(allyl glycidyl ether) (PEG-*b*-PAGE),

was synthesised via anionic ring-opening polymerisation using potassium alkoxide/naphthalenide as the initiator to give a block copolymer that contained a hydrophilic PEG block with a weight fraction of 30 % and a hydrophobic block with terminal allyl pendant units. This was formulated to form compartmental self-assembled nanoparticles (Fig. 31), with transmission electron microscopy (TEM) analysis revealing the formation of characteristic hollow nanoparticles with a well-defined spherical morphology, exhibiting a mono-modal distribution of particles of $ca\ 150 \pm 32\text{ nm}$, with a wall thickness estimated to be $4 \pm 1\text{ nm}$ (Fig. 31B).

As a reactive species, it is important to keep the balance of solution stability and fast reaction kinetics. We have previously reported that the tetrazine used in this study undergoes a fast reaction with poly(allyl glycidyl ethers) in water. Upon treatment of the nanoparticles with a water-soluble and hydrophilic tetrazine, the polymer backbone was rapidly modified, with dynamic light scattering studies revealing a dramatic reduction in diameter from 150 nm to 30 nm after four hours incubation (Figure 2a). The reaction will be slow initially since that the hydrophilic tetrazine has to reach to the hydrophobic layers, but following the modification, the rate will increase, as the membrane becomes more and more hydrophilic. TEM measurement confirmed that a population of micelles was generated with a uniform diameter of $30 \pm 5\text{ nm}$ (Fig. 31C, and 31D). These changes take place due to the modification of the hydrophobic moieties, arising from the conversing of the hydrophobic moieties into hydrophilic side chains (due to the nature of the tetrazine used and the high level of polymer modification). In support of these observations, the zeta-potential values of the NP exhibited a dramatic change from 0.12 mV to 24.10 mV following tetrazine modification.

The IC_{50} values of the NPs and tetrazine against the PC3 cells were determined as 5 mg ml^{-1} and $100\text{ }\mu\text{M}$ respectively, indicating relatively low cytotoxicity. To investigate cell internalisation, FITC labelled **PEG-*b*-PAGE** (**FITC-PEG-*b*-PAGE**) was prepared. The self-assembled NPs showed a time-dependent increase in fluorescence intensity in cells with an enhanced internalisation capacity in the presence of tetrazine as evidenced by flow cytometry and cell imaging.

To explore the cargo release profile of the nanoparticles upon the addition of tetrazine, nanoparticles loaded with Doxorubicin (DOX) were prepared (the DOX loading efficiency and dimensions of the DOX-loaded NPs are shown in table X). For measurement of release profiles, DOX loaded NPs (3 mg ml^{-1} in PBS) were dialysed (MWCO 20 kDa) against PBS (pH = 7.4, 10 mM) at $37\text{ }^{\circ}\text{C}$ and tetrazine ($50\text{ }\mu\text{M}$, 4 equiv. to allyl ether units) was introduced and the release of doxorubicin was monitored spectrophotometrically at 485 nm. The triggered

nanoparticles exhibited full cargo release, with the release profile mirroring the reduction in particle diameter (see Fig. 32b. In the absence of tetrazine, the amount of liberated DOX was negligible over 48 h, while in the presence of tetrazine, the release was rapid, with almost quantitative release over 10 hours. The trigger release of DOX was attributed to the gross morphological changes occurring to the polymer. Hydrophobic DOX is encapsulated in the membrane of the vesicles. The reaction of tetrazine with the allyl group will cause vesicles collapse rapidly as the global properties change quickly, but small hydrophobic pockets were still binding DOX release.

DOX loading Conc. (μM)	d (nm)	PDI	Encapsulation Efficiency (%)
5	150 ± 24	0.112	22 ± 4.1
20	145 ± 21	0.107	32 ± 3.6
50	160 ± 36	0.098	35 ± 6.2
100	178 ± 39	0.126	30 ± 4.1

Table X: Characterisation of the prepared DOX-loaded nanoparticles. (d = hydrodynamic diameter, PDI = polydispersity index, n=3)

Cell viability studies were undertaken in the presence of DOX loaded NPs, with and without the tetrazine trigger using PC3 cells stained with the vital cell stain CellTracker™ green. After 72 hours cell viability was quantified by flow cytometry using propidium iodide as a live/dead cell discriminator. The proportions of viable and non-viable cells were also evaluated as shown in Figure 33 with DOX loaded NPs by flow cytometry. The 1 and 6 μM DOX-NP's were non-cytotoxic over 72 hours in the absence of tetrazine, while the 18 μM DOX-NPs exhibited some cytotoxicity (87% cell viability compared to 95% for the control). These DOX loaded nanoparticles demonstrated significant effects on the cells following the introduction of the tetrazine, which triggered nanoparticle collapse and DOX release, resulting in cell death in a dose dependent manner. As shown in Fig. 33, upon the addition of a trigger tetrazine, a cytotoxic effect was observed for all concentrations with the 18 μM DOX loaded NP's resulting in 98% of cells taking up the stain propidium iodide (which is excluded by the plasma membrane in healthy cells) – an indication of cell death. In addition to triggering cargo release the tetrazine reaction also promoted cellular uptake, resulting in DOX-loaded nanoparticles increasingly entering cells as the reaction progressed.

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CLAIMS

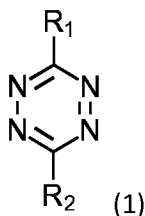
1. A kit for the administration and activation of a masked active agent, the kit comprising a masked active agent and a trigger, the masked active agent comprising:

- 5 i) an active agent connected to a masking moiety; or
 ii) an active agent enclosed within a cage moiety;

wherein the trigger is configured to release the active agent from the masking moiety or the cage moiety, and wherein the trigger is a tetrazine, and the masking moiety comprises a monovinyl ether and the cage moiety comprise a monovinyl ether or an allyl group.

2. The kit according to claim 1, wherein the masking moiety or the cage moiety comprise a phenolic monovinyl ether.

3. The kit according to either one of claims 1 and 2, wherein the tetrazine has the general structure:



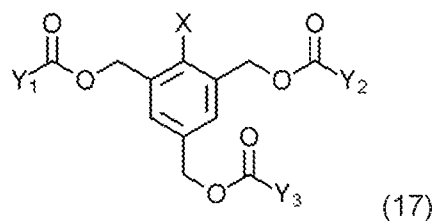
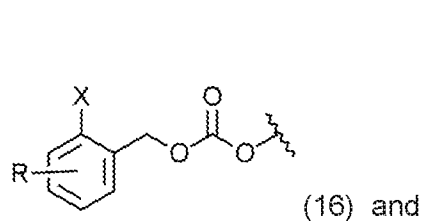
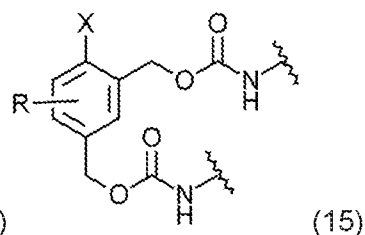
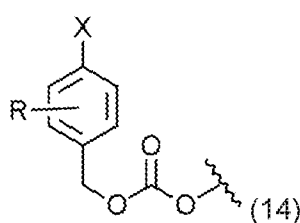
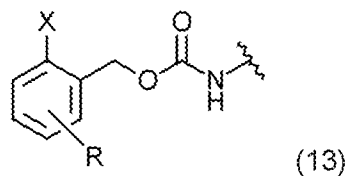
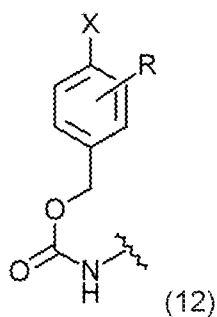
wherein R₁ and R₂ are weak electron withdrawing groups.

4. The kit according to claim 3, wherein R₁ and R₂ are chosen from pyridines, diazines, aryl halides, aryl ketones, aryl aldehydes or aryl esters.

5. The kit according to any one preceding claim where the masked active agent comprises an active agent connected to a masking moiety, wherein the active agent is directly linked to the masking moiety.

6. The kit according to claim 5, wherein the active agent comprises a hydroxyl group and the oxygen of the hydroxyl group of the active agent is connected to a vinyl group to thereby form the monovinyl ether masking moiety.

7. The kit according to any one of claims 1 to 4 where the masked active agent comprises an active agent connected to a masking moiety, wherein the active agent is indirectly linked to the masking moiety via a linker.
8. The kit according to claim 7, wherein the linker comprises a hydroxyl group and the oxygen of the hydroxyl group of the linker is chemically bonded to a vinyl group to thereby form the monovinyl ether masking moiety.
9. The kit according to either one of claims 7 and 8, wherein the linker comprises a polymer.
10. The kit according to any one of claims 7 to 9, wherein the linker comprises a bridge and a polymer extending away from the bridge, and wherein the bridge connects the active agent to the masking moiety.
11. The kit according to any one of claims 7 to 10, wherein the linker is selected from the group of linkers comprising:

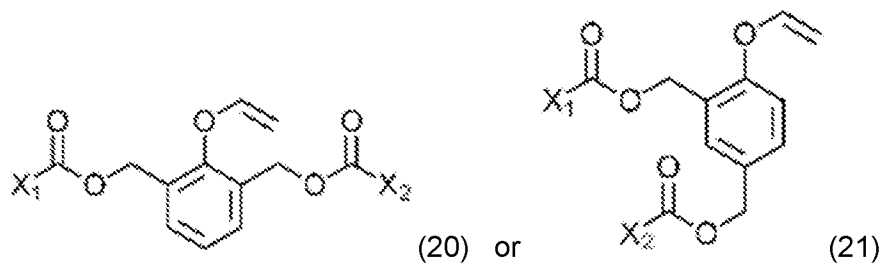


wherein X is a monovinyl ether masking moiety;

Y₁, Y₂ and Y₃ are active agents;

and R is selected from H, C₁-C₂₀ alkyl, C₁-C₂₀ alkenyl, C₁-C₂₀ alkynyl, a polymer such as polyethylene glycol, poly(methyl methacrylate), poly(oxazoline), C₁-C₂₀ hydroxyl, halide (e.g. F, Cl, Br, I), C₁-C₂₀ ether, C₁-C₂₀ carboxylic acid, C₁-C₂₀ amide, C₁-C₂₀ amine, C₁-C₂₀ aldehyde, C₁-C₂₀ ketone.

12. The kit according to any one of claims 9 to 11, wherein the polymer is configured to fragment when the monovinyl ether moiety is removed from the polymer, to thereby release the active agent.
13. The kit according to any one preceding claim, wherein the masked active agent comprises a targeting moiety.
14. The kit according to any one preceding claim, wherein the masked active agent is a masked fluorophore, wherein the masked fluorophore is a masked fluorescein, a masked naphthofluorescein, a masked resorufin, a masked 5(6)-carboxyfluorescein or a masked coumarin.
15. The kit according to any one of claims 1 to 13, wherein the active agent is a therapeutic active agent and the masked active agent is a prodrug of that therapeutic active agent, wherein the therapeutic active agent is fluorouracil, doxorubicin, camptothecin, clofarabine, fludarabine, cladribine, Etoposide, daunorubicin, or cis-platin.
16. The kit according to any one preceding claim, wherein the masked active agent comprises at least two masking moieties.
17. The kit according to any one preceding claim, wherein the masked active agent is linked to a further active agent via a common masking moiety, such that removal of the monovinyl ether of the masking moiety results in the release of the active agent and/or the further active agent, and wherein the common masking moiety has the structure:



wherein X_1 is the active agent, and X_2 is the further active agent or vice versa.

- 5 18. The kit according to any one preceding claim where the masked active agent comprises a cage moiety, wherein the masked active agent comprises a plurality of cage moieties.
19. The kit according to claim 18, wherein the cage moieties within the plurality of cage
- 10 moieties comprise polymers.
20. The kit according to claim 18 or claim 19, wherein the plurality of cage moieties form a superstructure that encapsulates the active agent.
- 15 21. The kit according to claim 20, wherein the superstructure is in the form of nanoparticles, a hydrogel, fibres, vesicles or microcapsules.
22. A masked active agent comprising an active agent connected to a masking moiety, an active agent enclosed within a cage moiety; wherein the masking moiety comprises a monovinyl ether and the cage moiety comprise a monovinyl ether or allyl group moiety,
- 20 wherein the masked active agent is configured to release the active agent from the masking moiety or the cage moiety in the presence of a trigger, and wherein the trigger is a tetrazine.
- 25 23. The masked active agent according to claim 22 where the masked active agent comprises a masking moiety connected to an active agent, wherein the active agent is directly linked to the monovinyl ether moiety.
24. The masked active agent according to either one of claims 22 or 23 where the masked
- 30 active agent comprises a masking moiety, wherein the active agent is indirectly linked to the masking moiety via a linker.

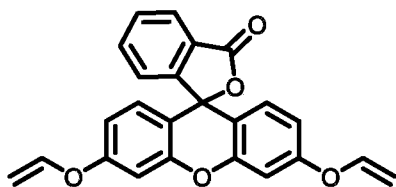
25. The masked active agent according to claim 24, wherein the linker comprises a polymer.
- 5 26. The masked active agent according to claim 24 or claim 25, wherein the linker is selected from the group of linkers comprising structures (12) to (16) as defined in claim 11.
- 10 27. The masked active agent according to any one of claims 22 to 26 further comprising a targeting moiety.
28. The masked active agent according to any one of claims 22 to 27, wherein the masked active agent is a masked fluorophore, and wherein the masked fluorophore is a masked fluorescein, a masked naphthofluorescein, a masked resorufin, a masked 5(6)-carboxyfluorescein or a masked coumarin.
- 15 29. The masked active agent according to any one of claims 22 to 27, wherein the active agent is a therapeutic active agent and the masked active agent is a prodrug of that therapeutic active agent, and wherein the therapeutic active agent is fluorouracil, doxorubicin, clofarabine, fludarabine, cladribine, Etoposide, daunorubicin or camptothecin.
- 20 30. The masked active agent according to any one of claims 22 to 29, wherein the masked active agent is linked to a further active agent via a common masking moiety.
- 25 31. The masked active agent according to any one of claims 22 to 30 where the masked active agent comprises a cage moiety, wherein the cage moiety comprises a plurality of branch elements extending from a core.
- 30 32. The masked active agent according to claim 31, wherein the core comprises a monovinyl ether or allyl group, the modification of which results in the release of the active agent from the cage moiety.
- 35 33. The masked active agent according to either one of claims 31 or 32, wherein at least some of the branch elements within the plurality of branch elements are cross-linked such that the active agent is restrained between the plurality of branch elements, and therefore, constitutes the masked active agent.

34. The masked active agent according to any one of claims 22 to 33 where the masked active agent comprises a cage moiety, wherein the masked active agent comprises a plurality of cage moieties, such that the plurality of cage moieties form a superstructure that encapsulates the active agent.
35. The masked active agent according to claim 34, wherein the superstructure forms a dendrimer structure, nanoparticles, a hydrogel, fibres, vesicles or microcapsules.
36. A pharmaceutical composition comprising a prodrug form of a therapeutic active agent, wherein the prodrug form of the therapeutic active agent comprises the therapeutic active agent and a monovinyl ether moiety.
37. The composition according to claim 36, wherein the prodrug is configured to release the therapeutic active agent upon reaction of the prodrug with a tetrazine trigger.
38. A method of modifying an active agent into a masked active agent comprising the steps of:
- (a) providing an active agent; and
 - (b) chemically linking the active agent to a vinyl group via a hydroxyl group of the active agent to form a monovinyl ether;
- wherein the monovinyl ether is configured to be converted to a hydroxyl group by a tetrazine trigger.
39. A method of modifying an active agent into a masked active agent comprising the steps of:
- (a) providing an active agent; and
 - (b) encapsulating the active agent with one or more caging moieties to form a masked active agent;
- wherein the caging moiety comprises a monovinyl ether configured to be removed from the caging moiety by a tetrazine trigger, or
- wherein the caging moiety comprises an allyl group configured to be modified by a tetrazine trigger,
- such that the active agent may be released from the masked active agent upon application of a tetrazine trigger.
40. Use of a tetrazine as a trigger for the release, in a physiological environment, of an active agent, wherein the active agent is connected to one or more monovinyl ether

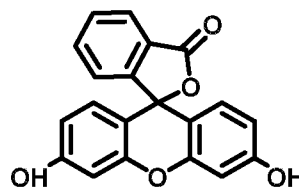
moieties before release to form a masked active agent, and wherein the one or more monovinyl ether moieties are removed from the active agent by the tetrazine during release.

- 5 41. Use of a tetrazine to remove one or more monovinyl ether groups during active agent synthesis, wherein the one or more monovinyl ether groups prevent hydroxyl groups on the substrate from reacting in one or more earlier steps of the synthesis.
- 10 42. Use of a tetrazine to selectively remove one or more monovinyl ether groups from a peptide comprising one or more monovinyl ether groups.
- 15 43. Use of the inverse electron-demand Diels-Alder reaction between a monovinyl ether and a tetrazine as a chemical tool for the release, in a physiological environment, of a substance administered as a masked active agent comprising a monovinyl ether.
- 20 44. Use of a tetrazine as a trigger for polymer fragmentation for a polymer linked to a monovinyl ether.
- 25 45. The use of claim 44, wherein the polymer comprises an active agent, and fragmentation of the polymer releases the active agent.
- 30 46. The use according to claim 44, wherein the polymer encapsulates an active agent and fragmentation of the polymer activated by the reaction of the tetrazine with the monovinyl ether releases the active agent.
47. Use of a tetrazine as an activator for modifying the hydrophobicity of a polymer comprising one or more allyl groups.
48. The use of claim 47, wherein the polymer forms aggregates in aqueous solution and modification of the hydrophobicity of the polymer results in a change in the morphology of the aggregates such that active agents encapsulated within the aggregates before activation by the tetrazine are released.

a)



Vinylfluorescein
Non-Fluorescent



Fluorescein
Fluorescent

b)

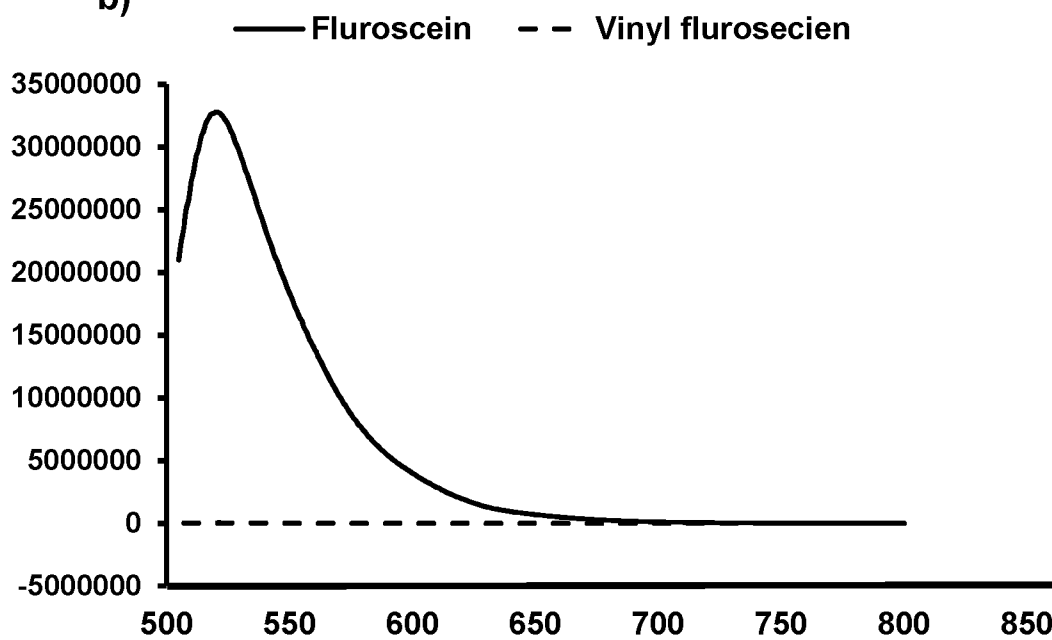


Fig. 1

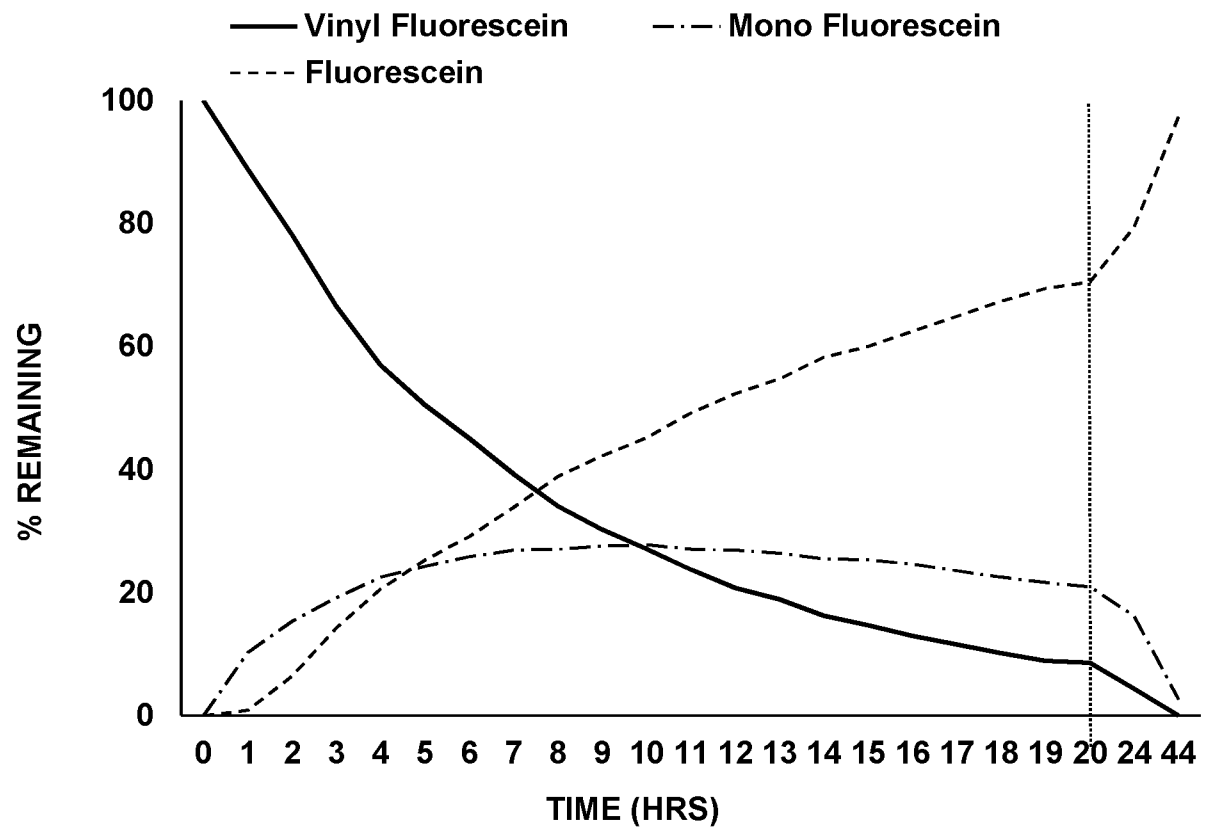


Fig. 2

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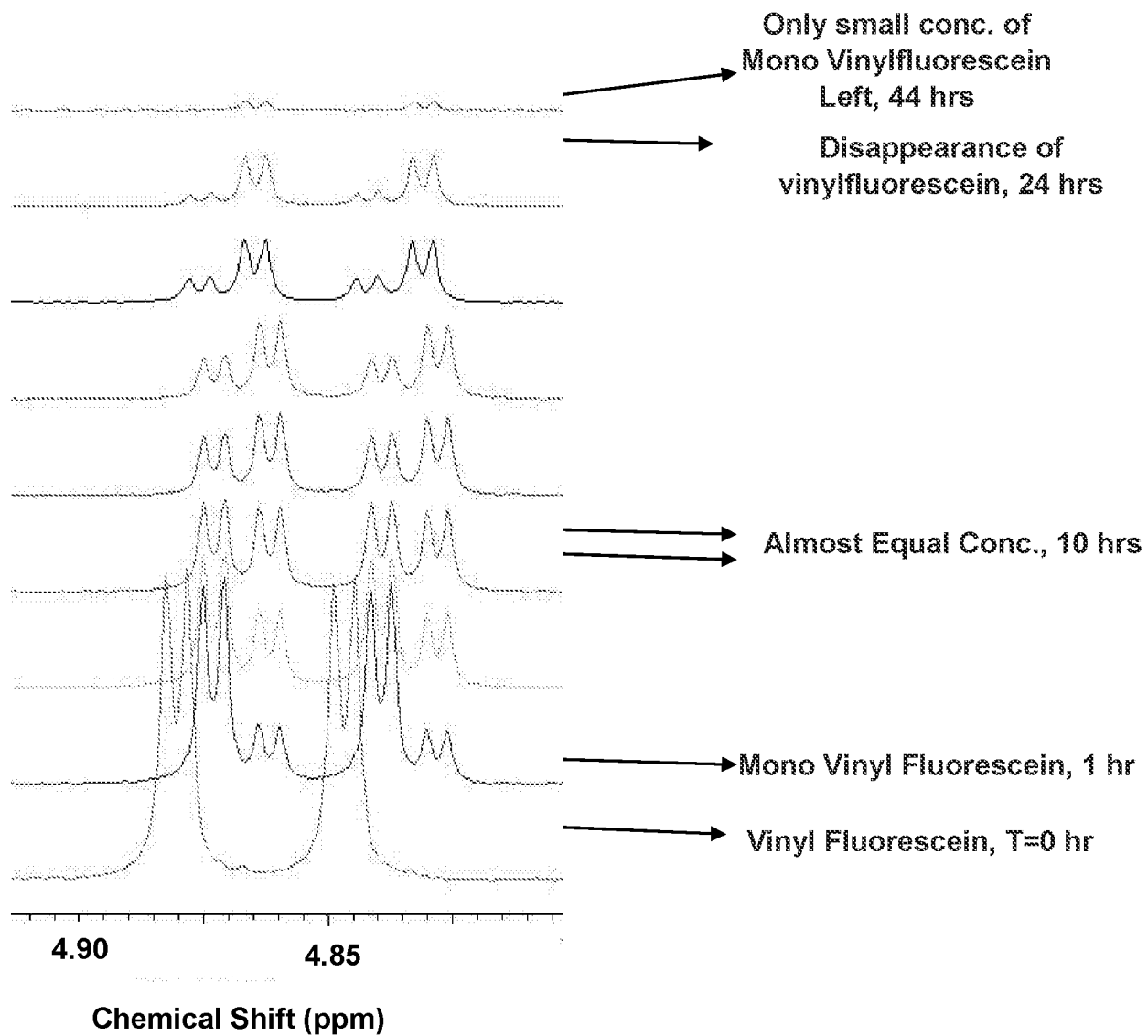


Fig. 3

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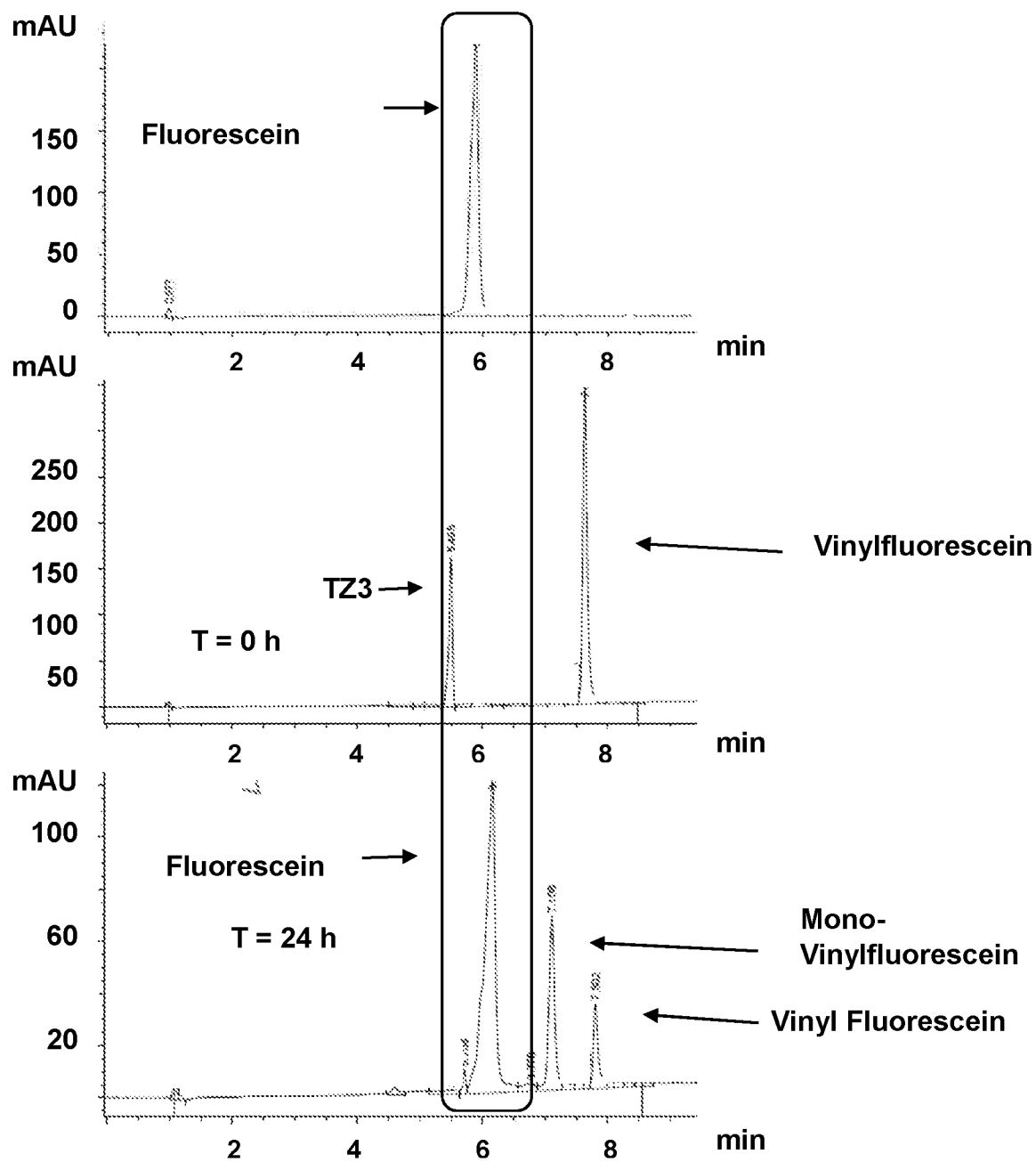


Fig. 4

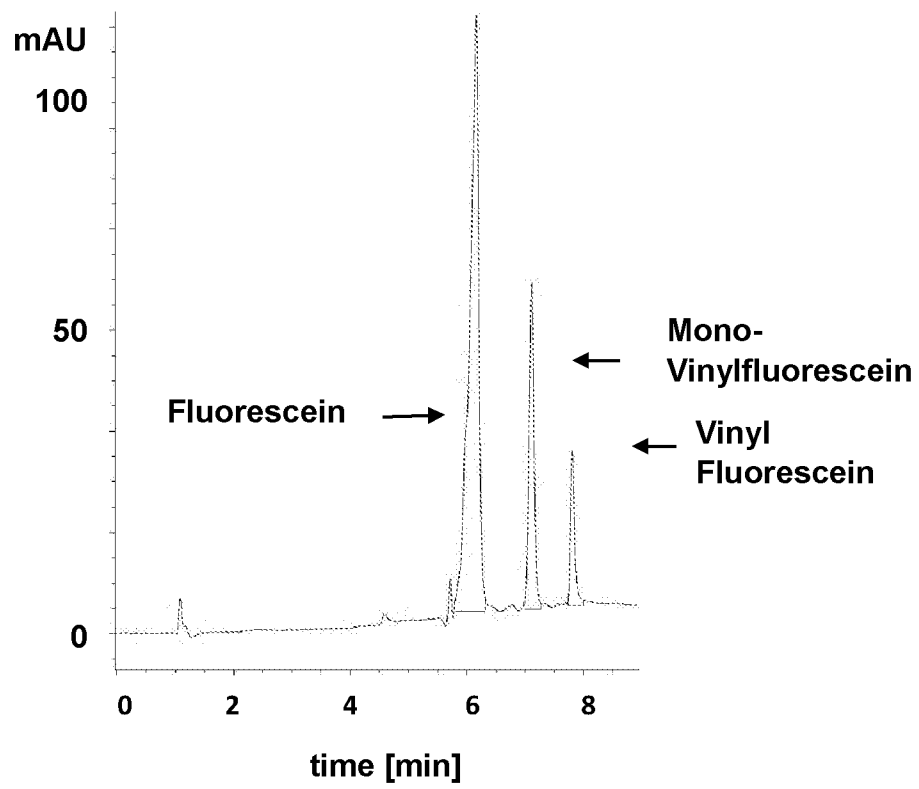


Fig. 5

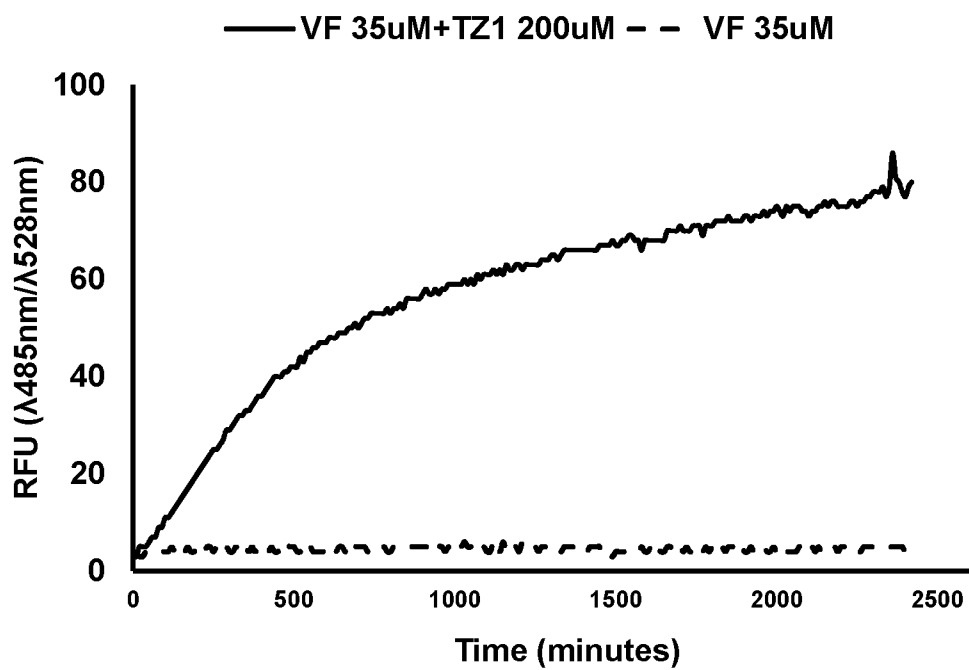


Fig. 6

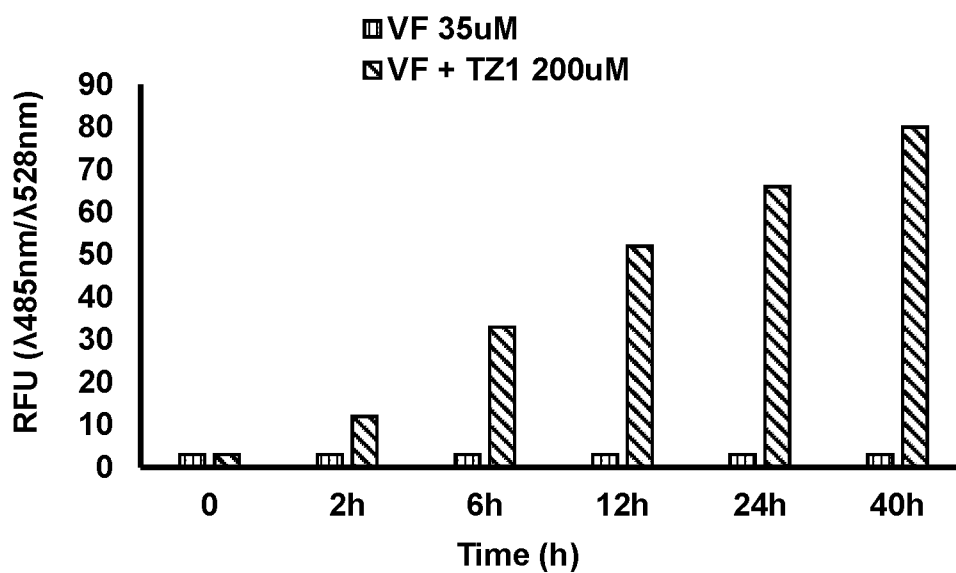


Fig. 7

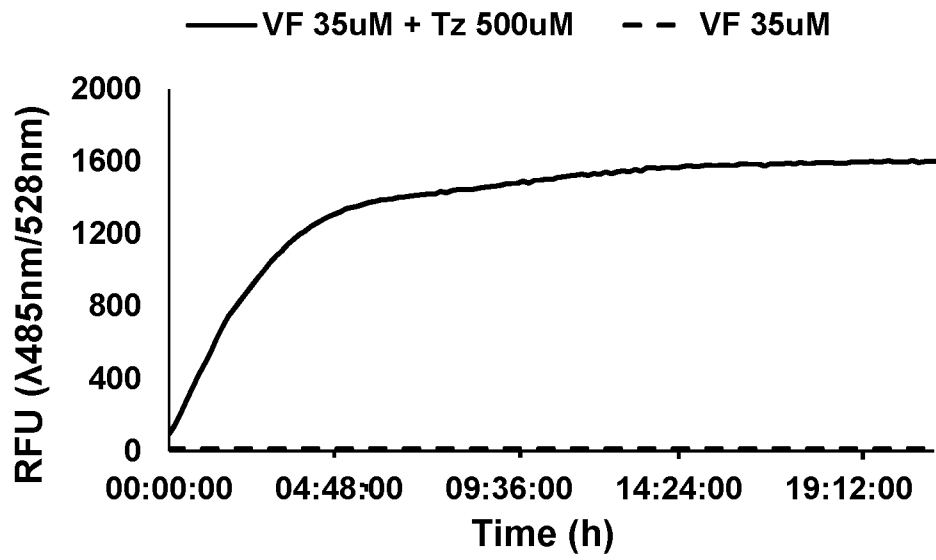


Fig. 8

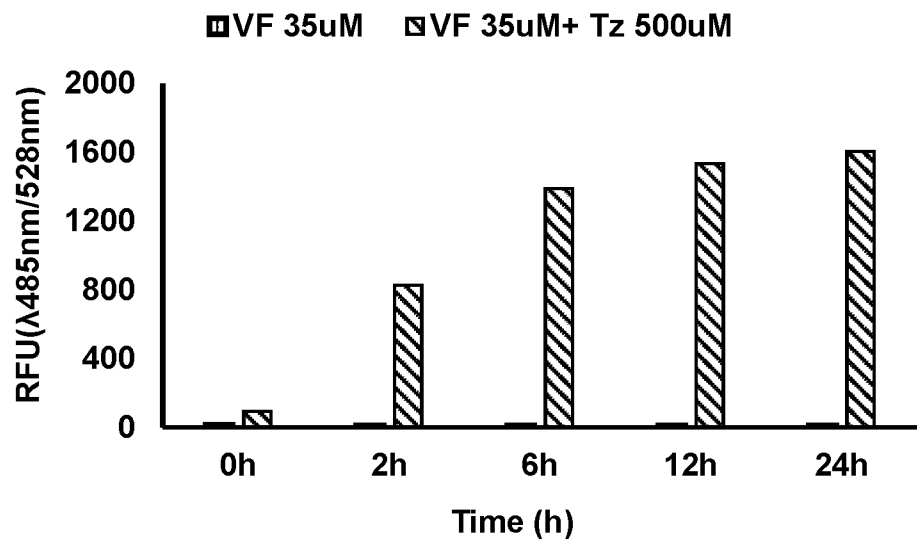


Fig. 9

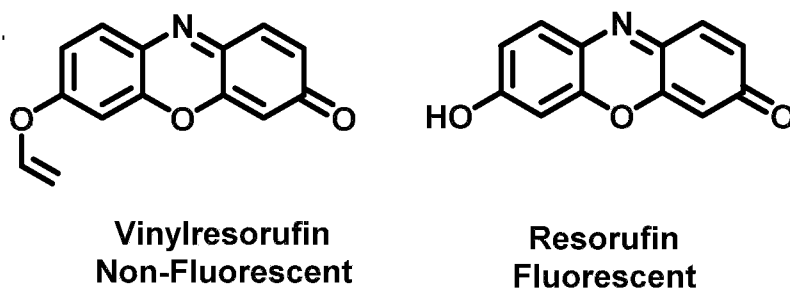


Fig. 10a

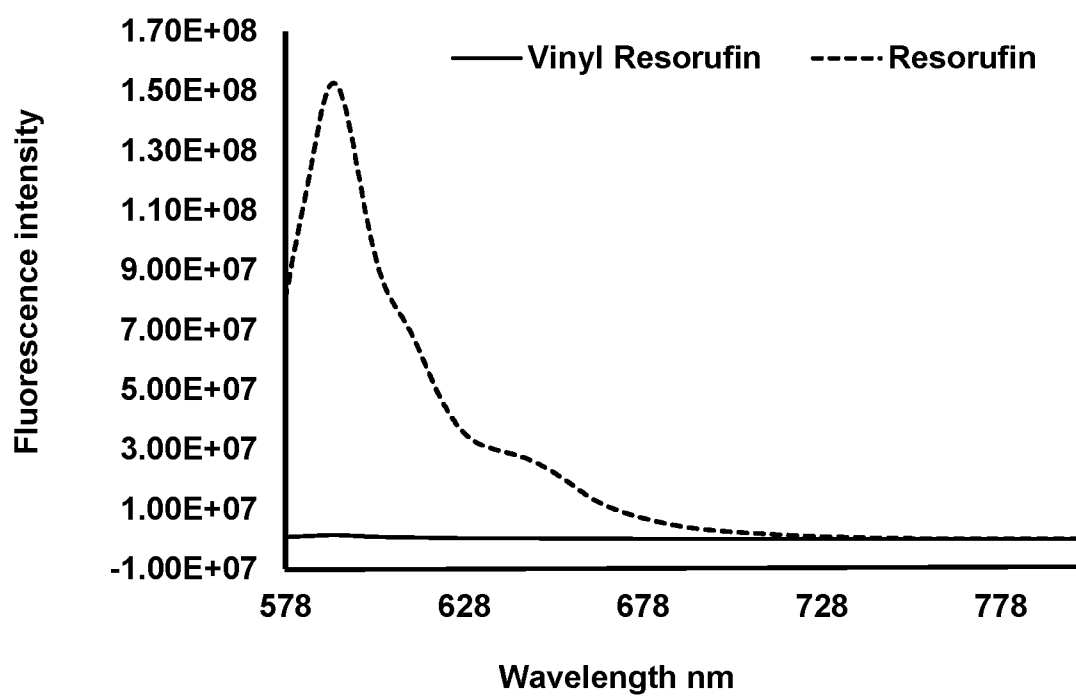


Fig. 10b

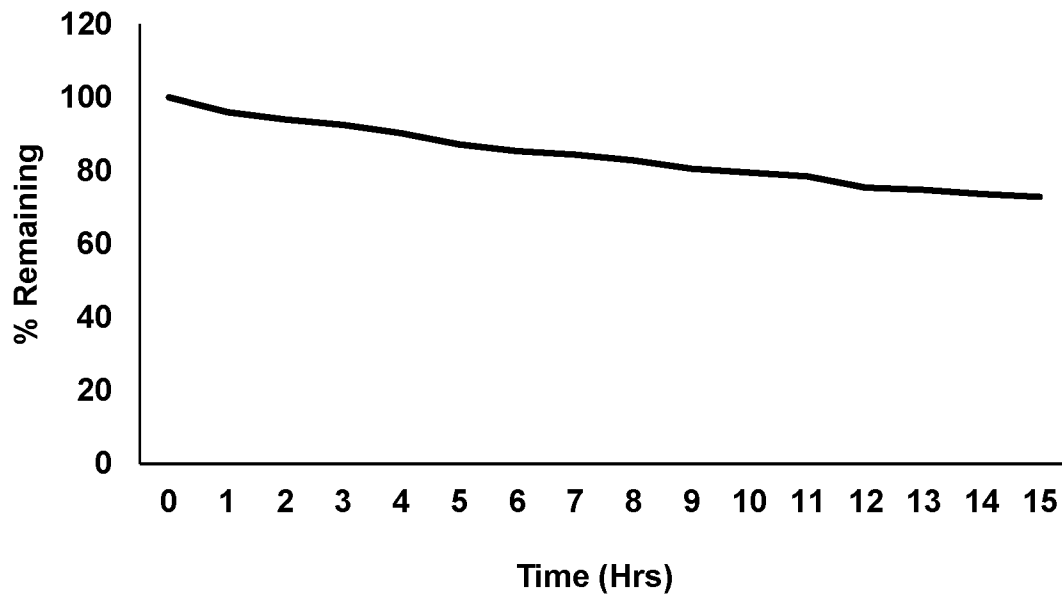


Fig. 11

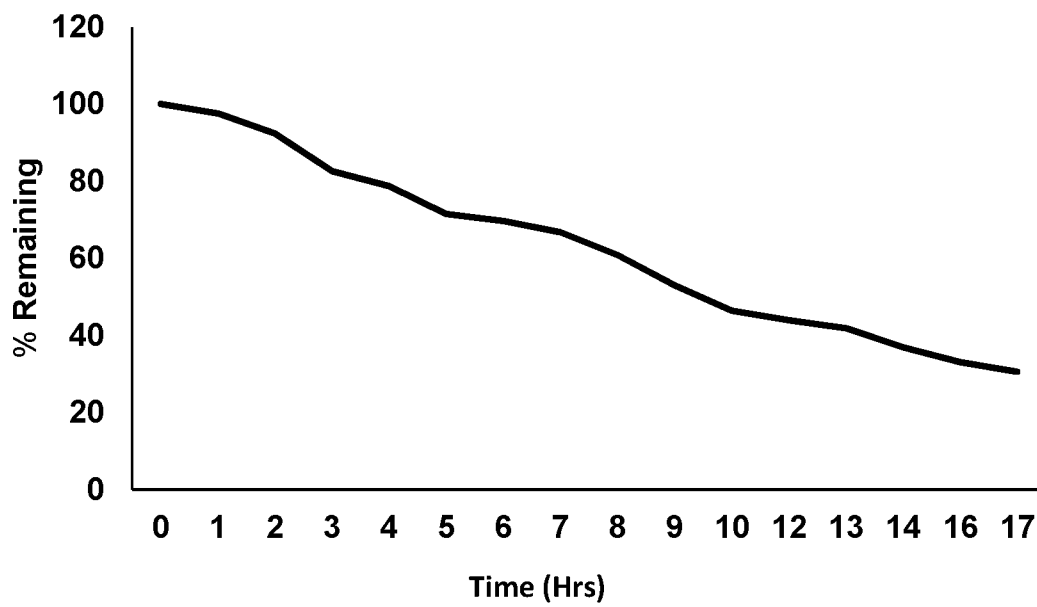


Fig. 12

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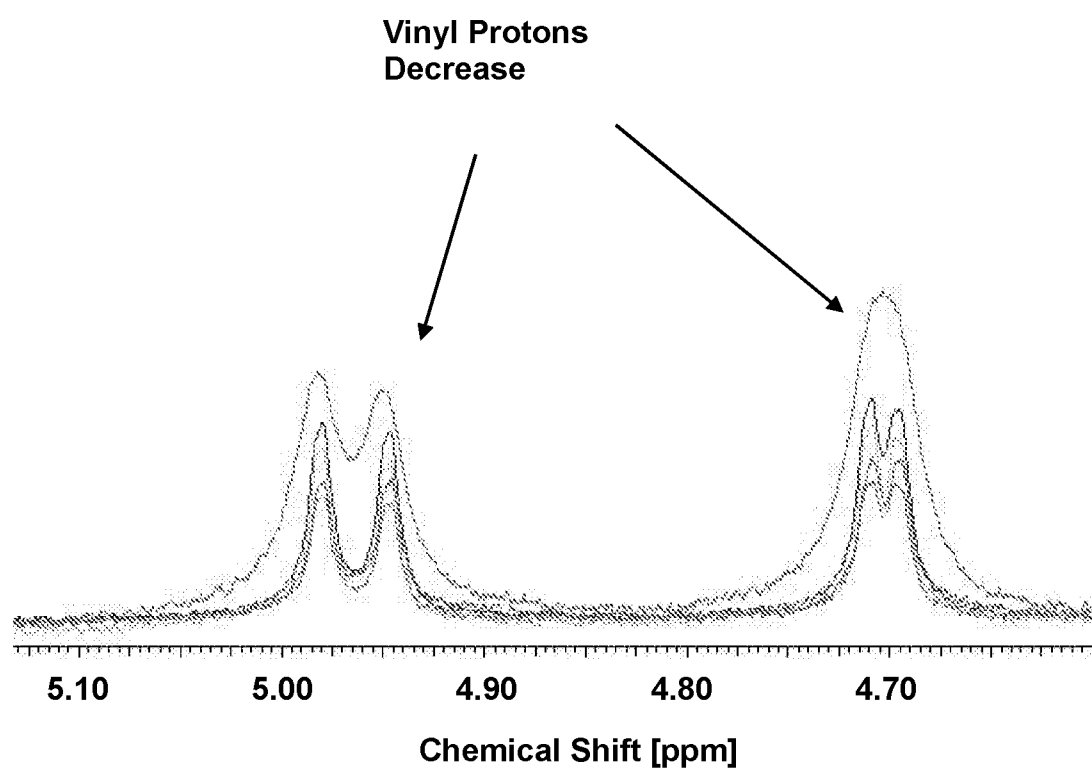


Fig. 13

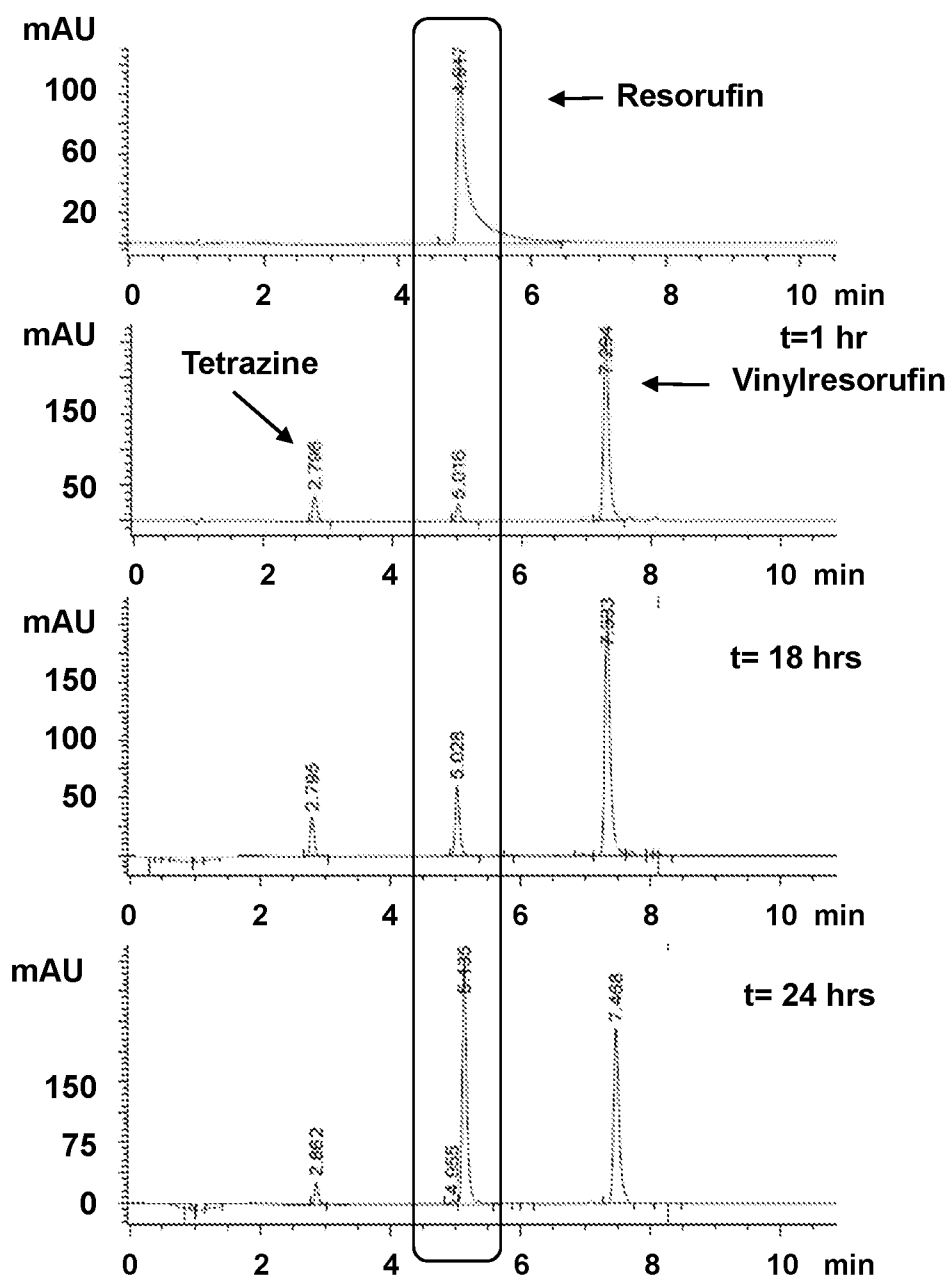


Fig. 14

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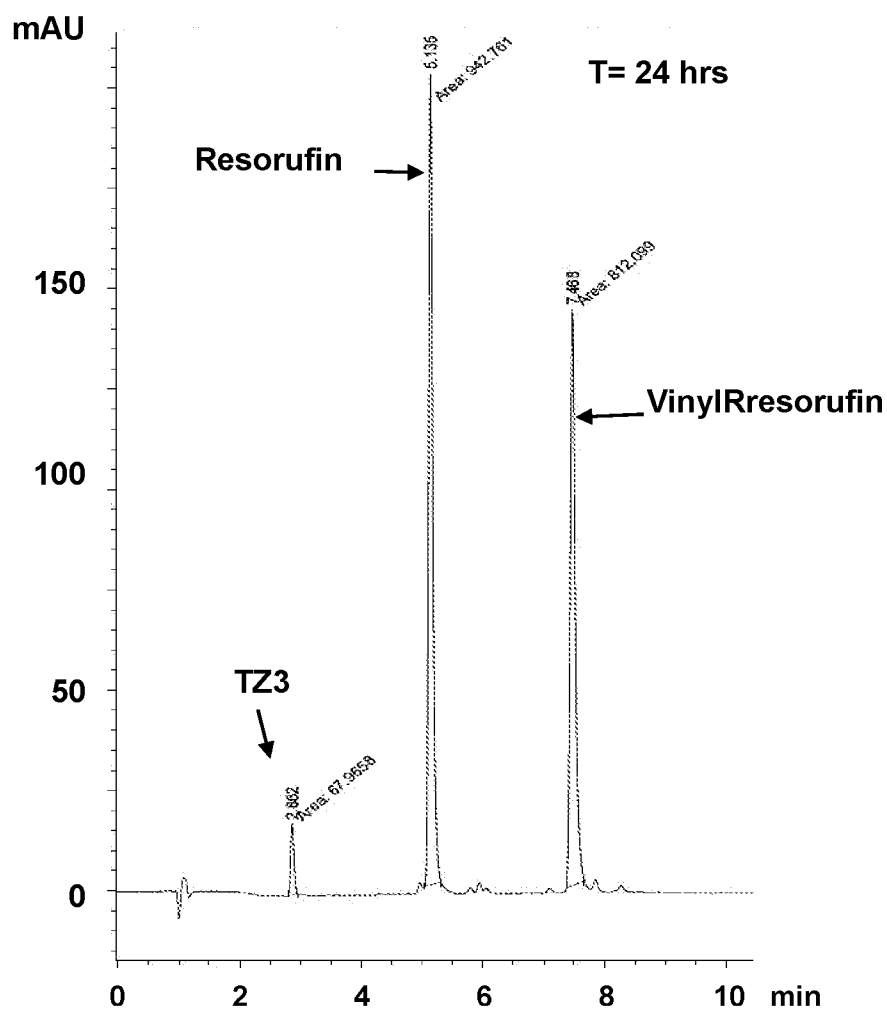


Fig. 15

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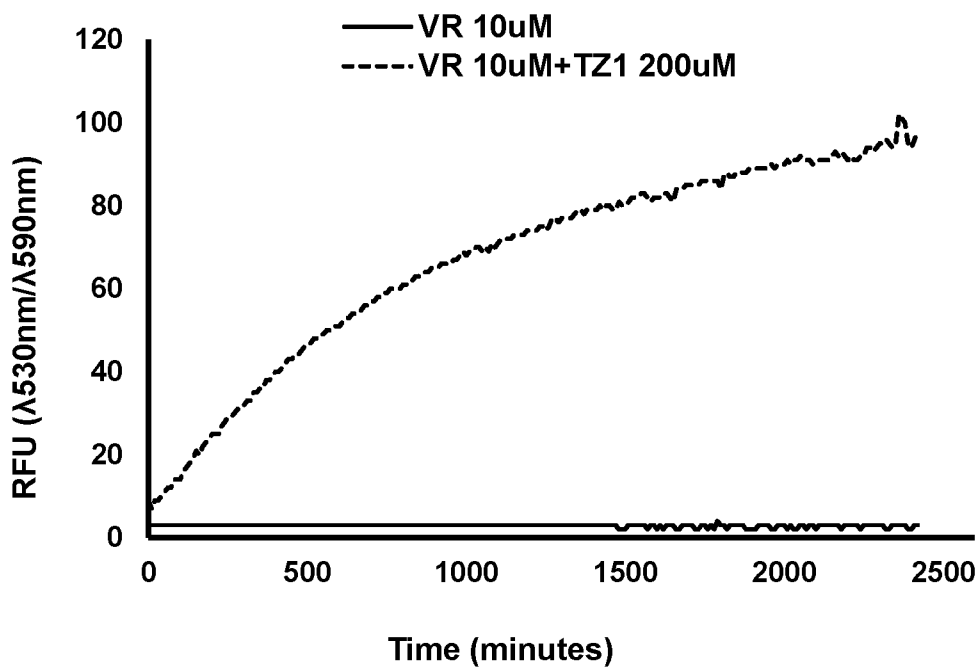


Fig. 16

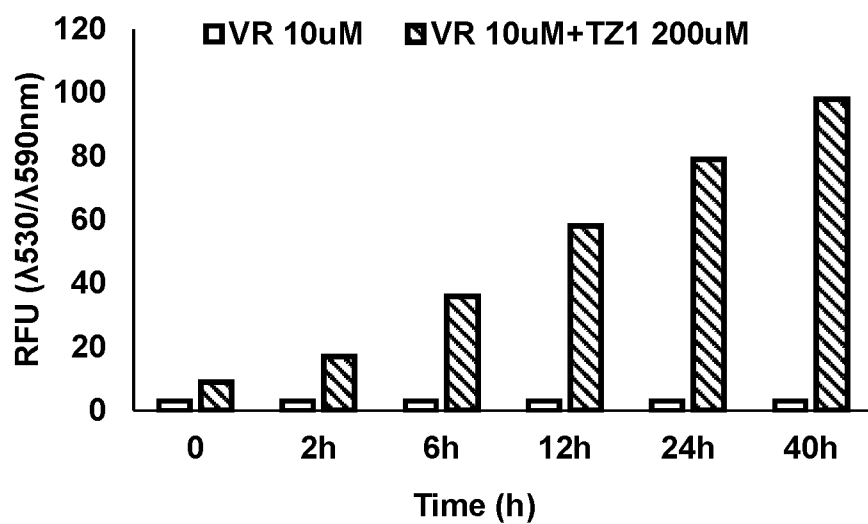


Fig. 17

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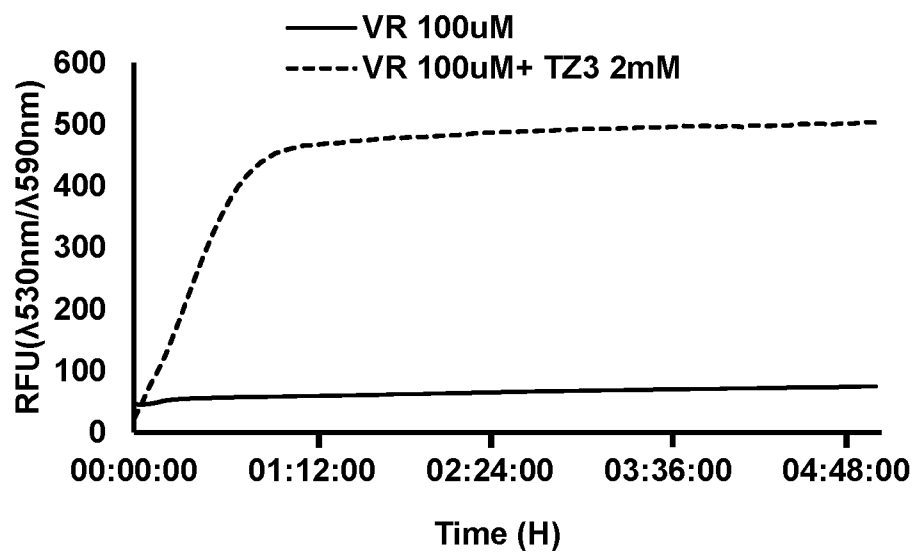


Fig. 18

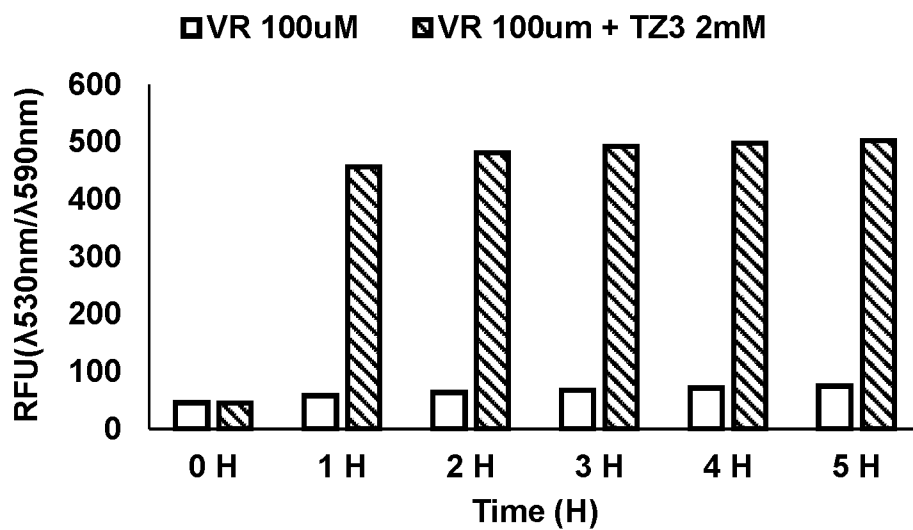


Fig. 19

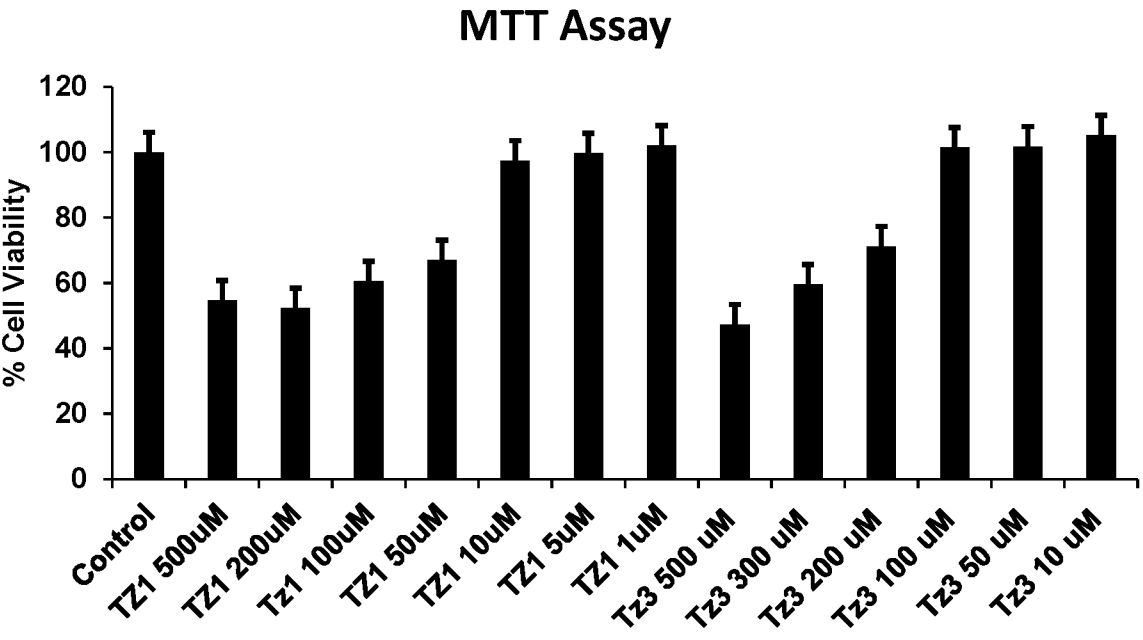


Fig. 20

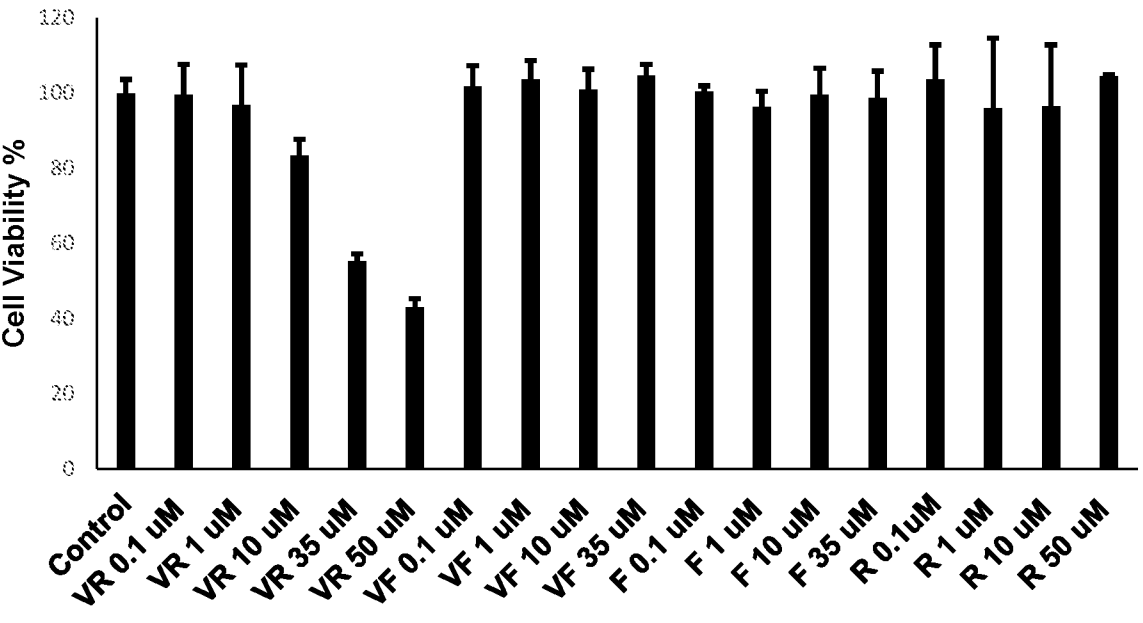


Fig. 21

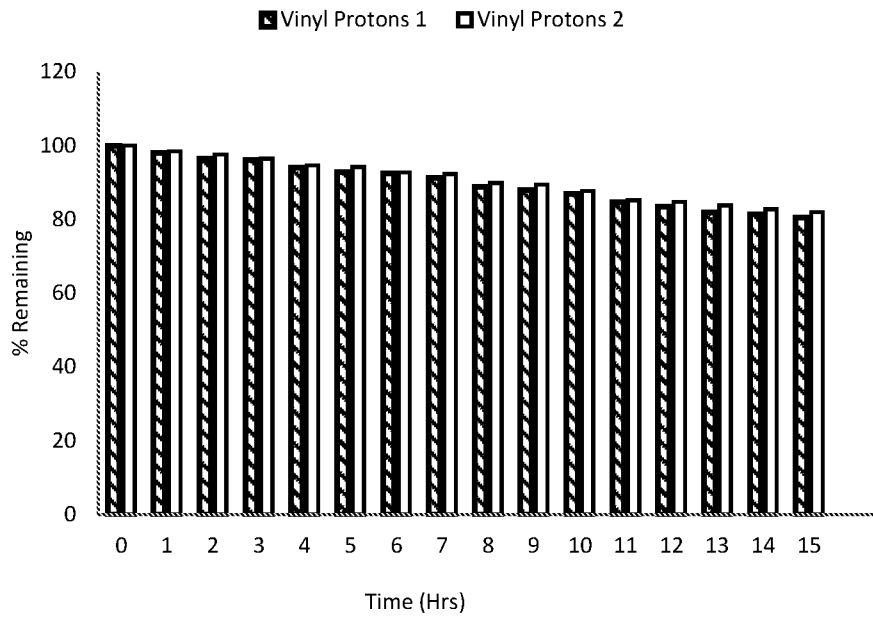


Fig. 22

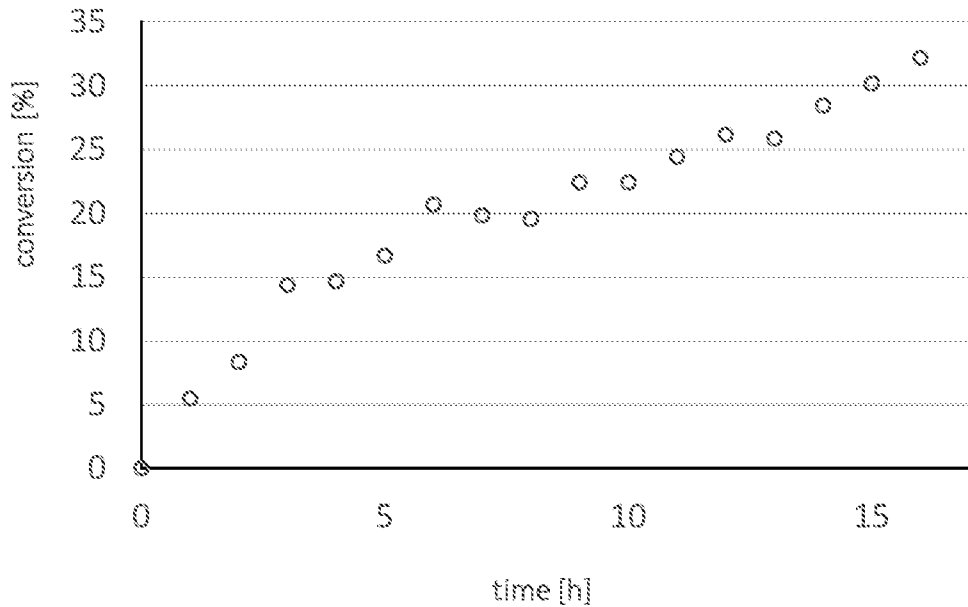


Fig. 23

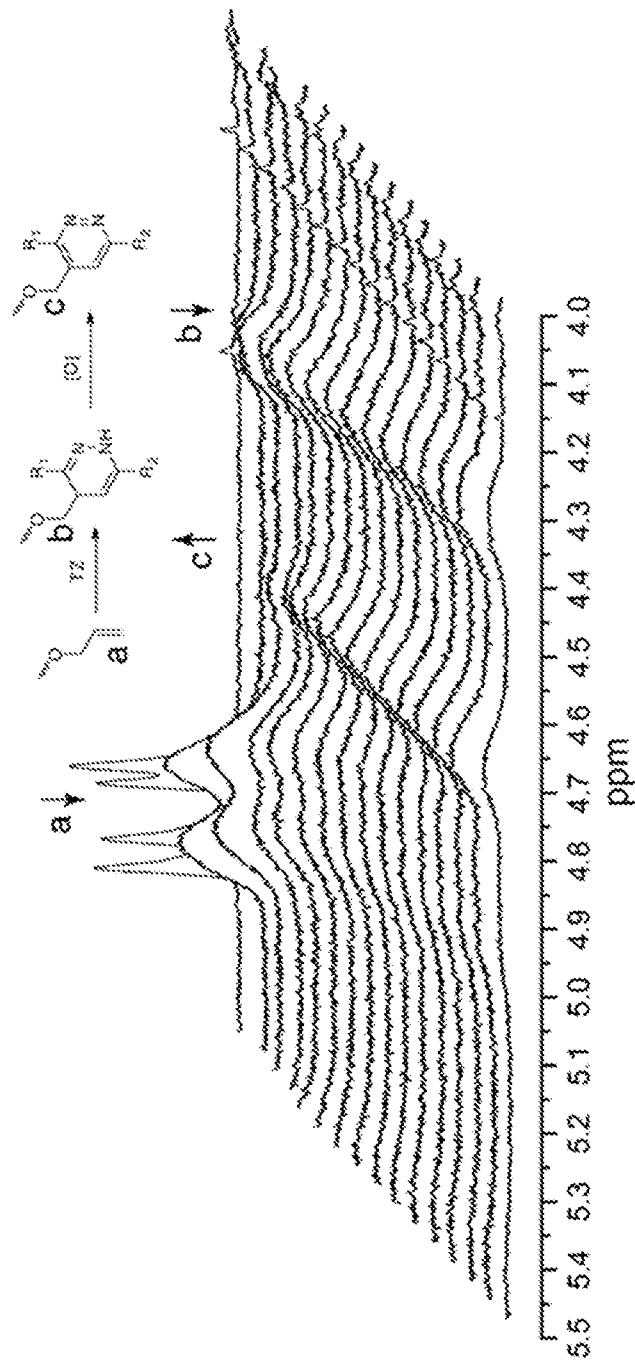


Fig. 24

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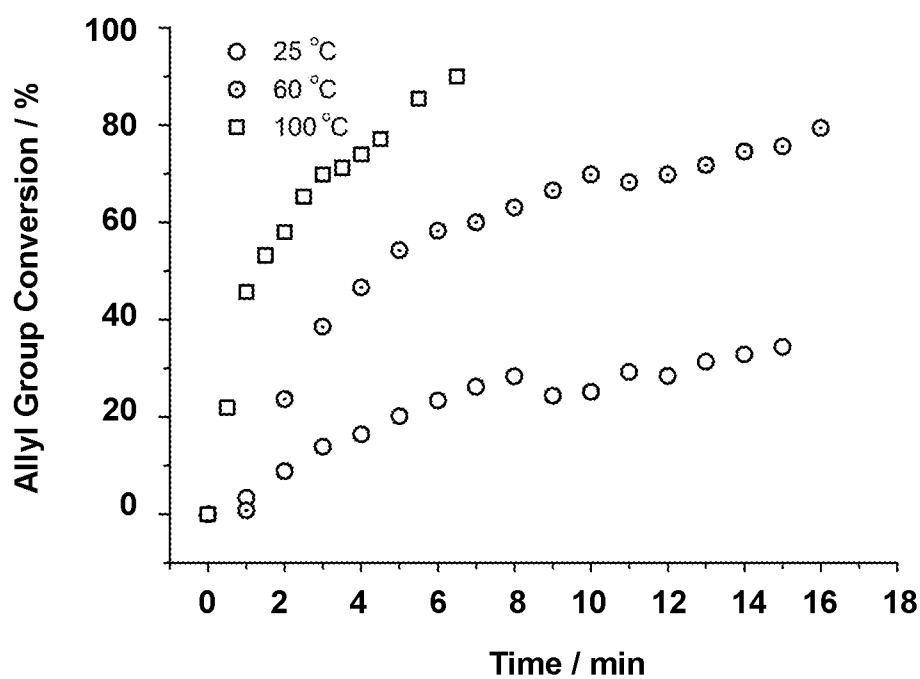


Fig. 25

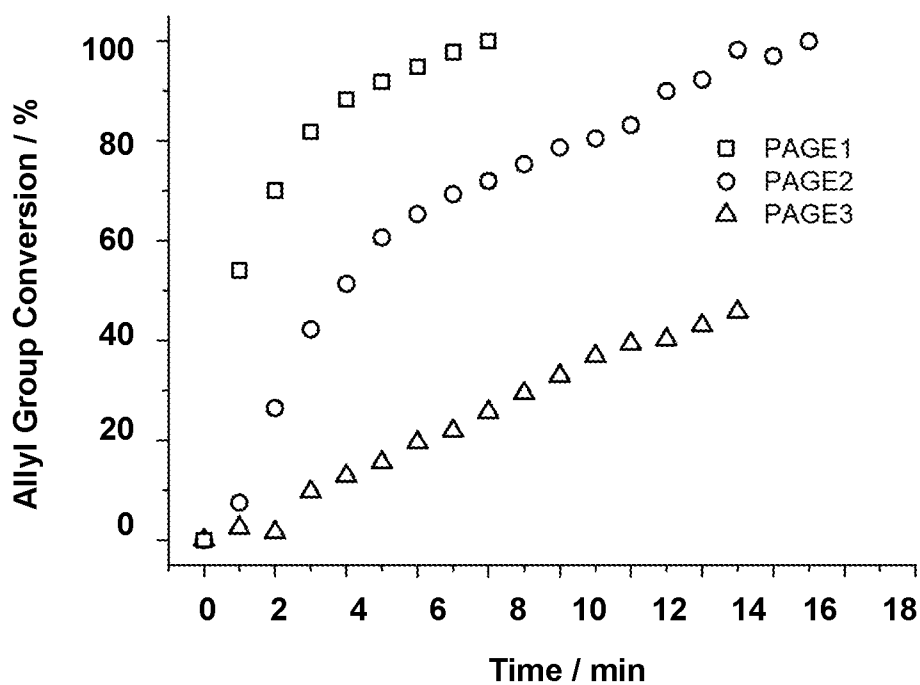
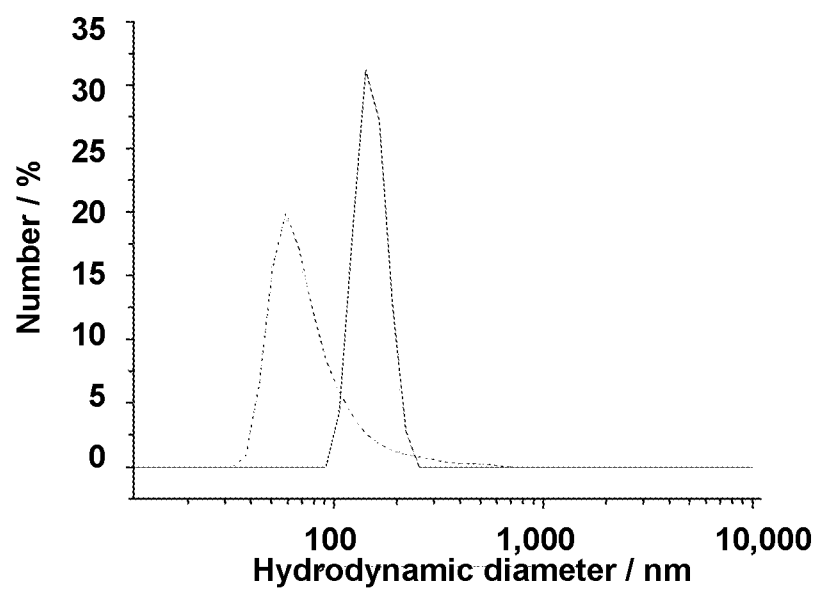


Fig. 26

A



B

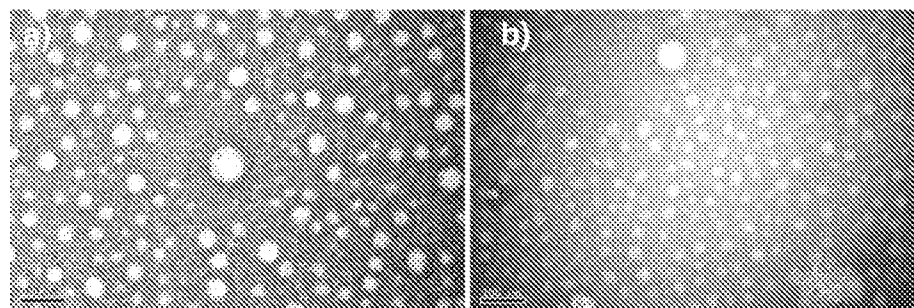


Fig. 27

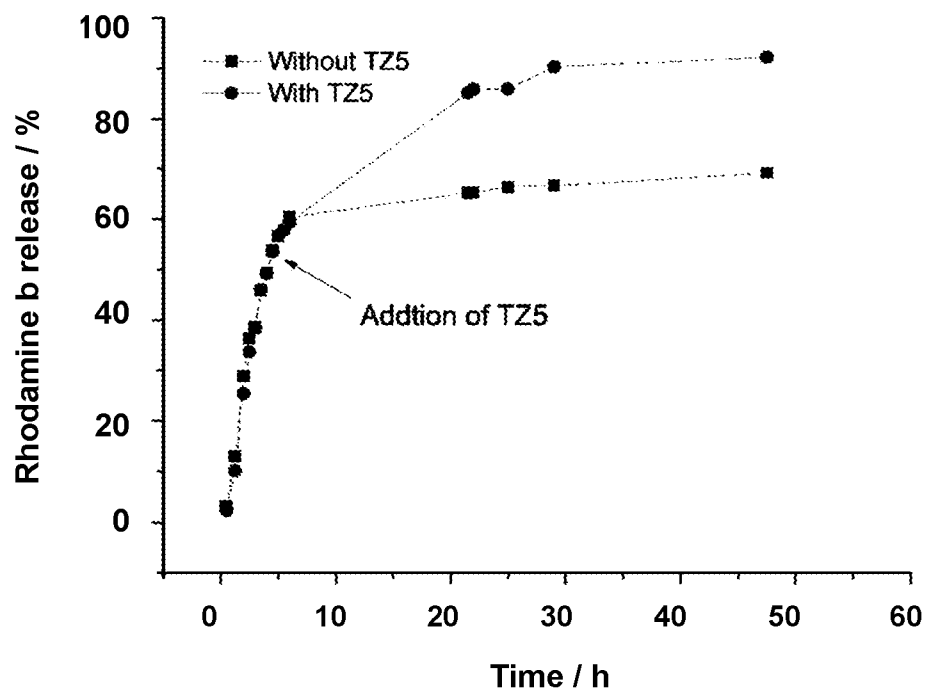


Fig. 28

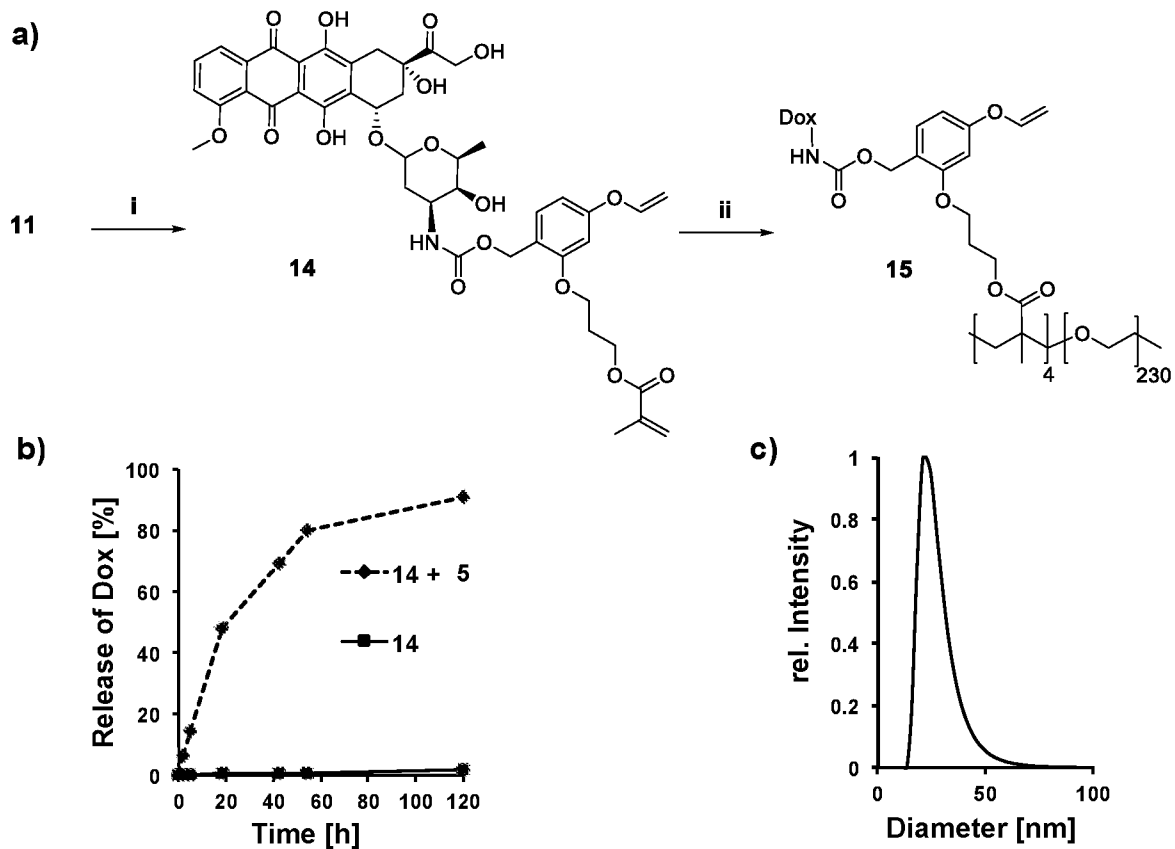


Fig. 29

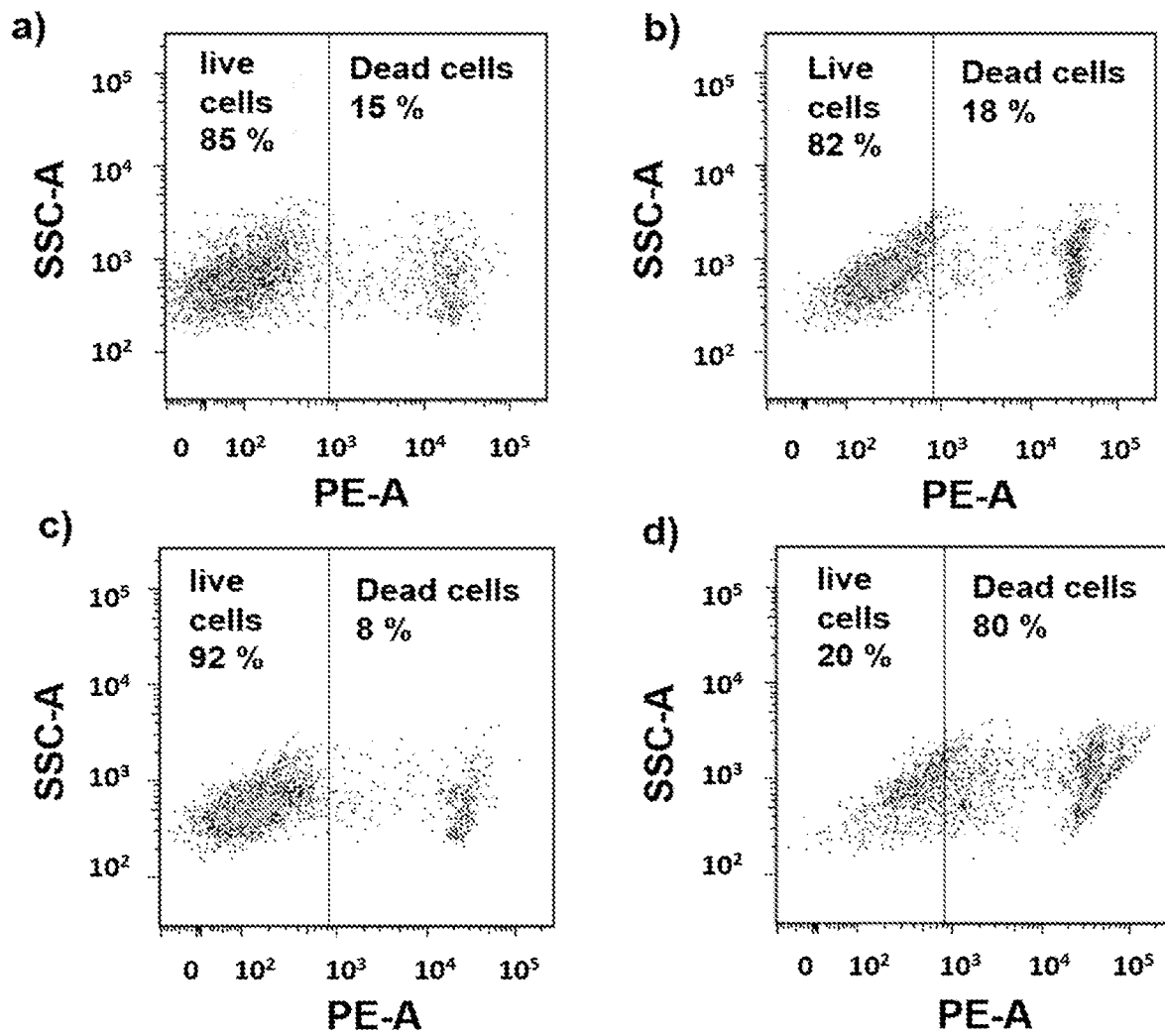


Fig. 30

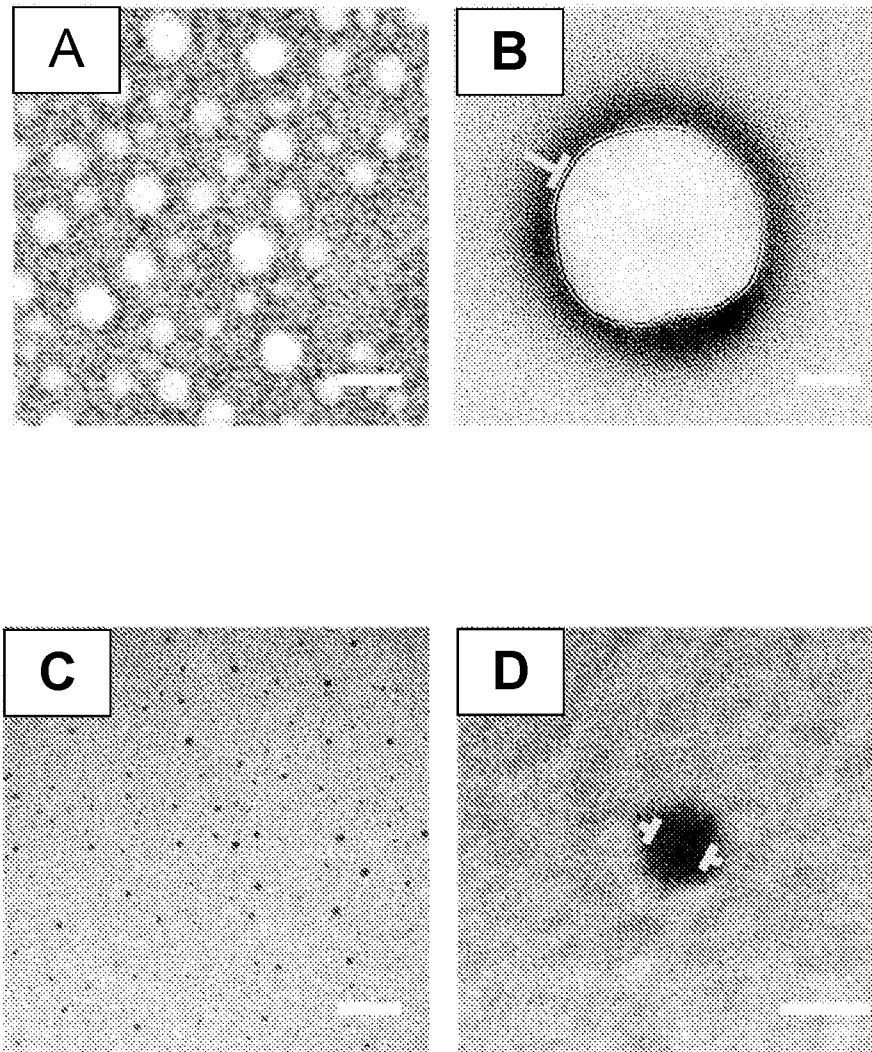


Fig. 31

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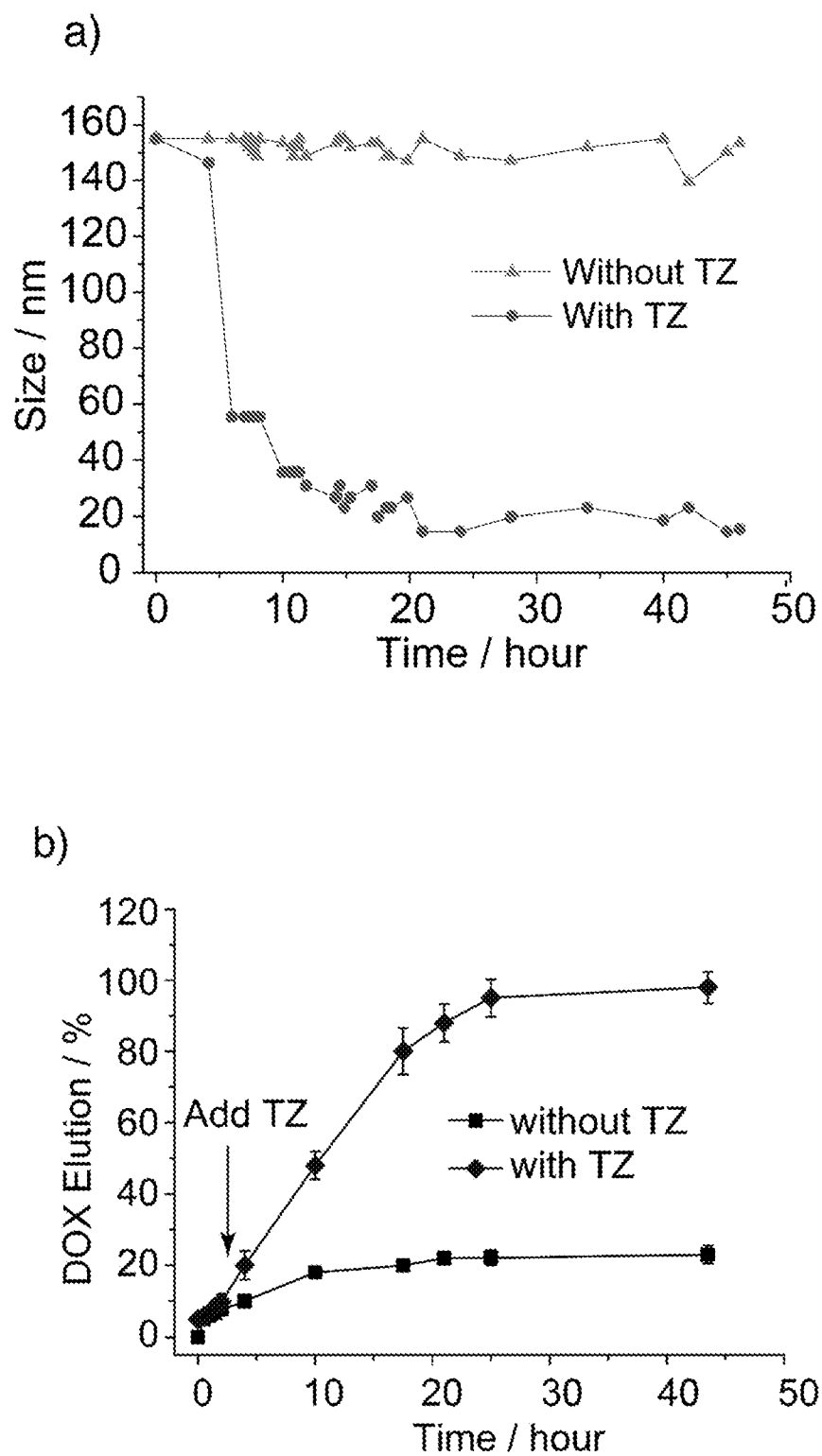


Fig. 32

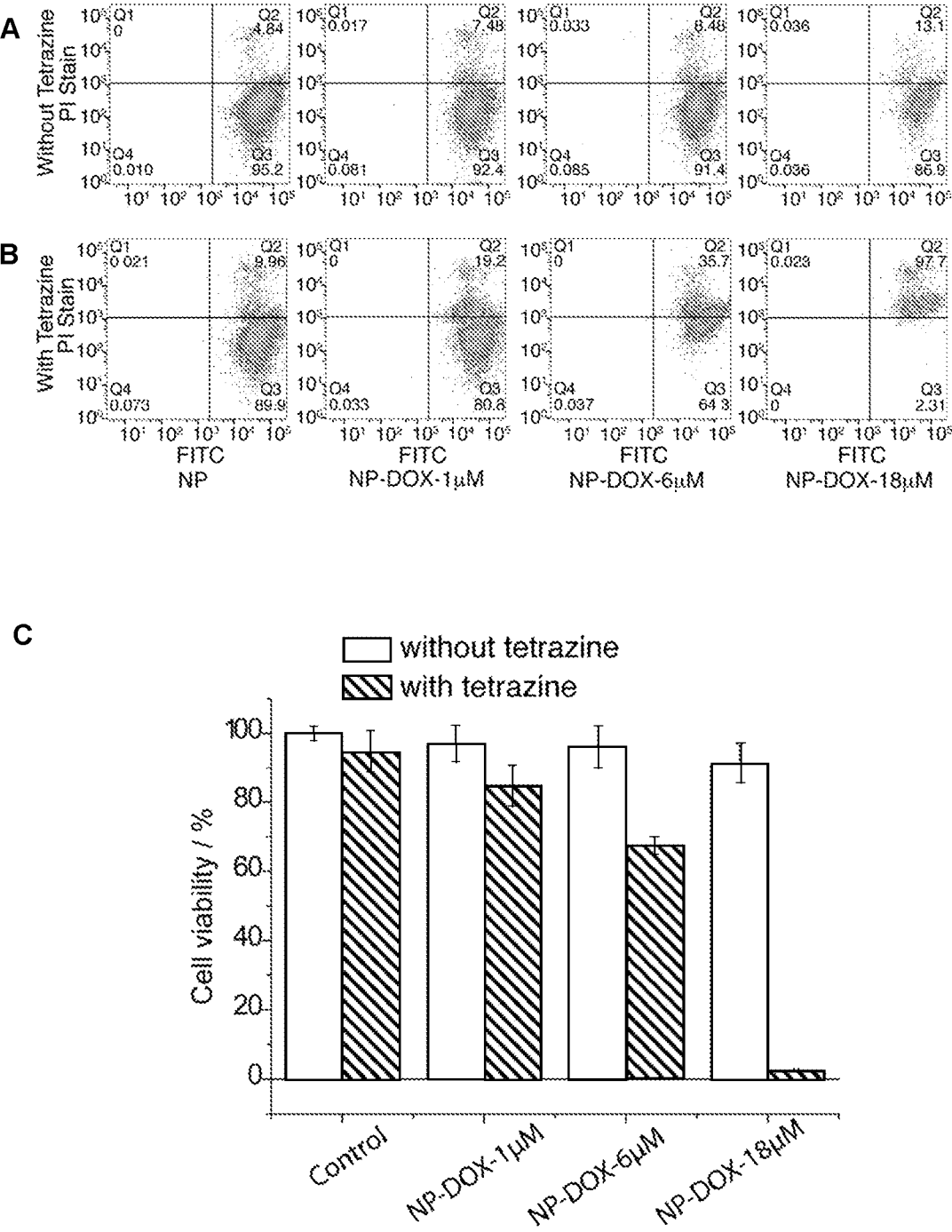


Fig. 33

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052895

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/54 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/081299 A1 (TAGWORKS PHARMACEUTICALS B V [NL]) 30 May 2014 (2014-05-30) page 37	47,48
A	WO 2014/081301 A1 (TAGWORKS PHARMACEUTICALS B V [NL]) 30 May 2014 (2014-05-30) claim 1 ----- -/-	1-48



Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search

22 November 2016

Date of mailing of the international search report

29/11/2016

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Langer, Miren

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AKIYUKI HAMASAKI ET AL: "Two Novel 1,2,4,5-Tetrazines that Participate in Inverse Electron Demand Diels-Alder Reactions with an Unexpected Regioselectivity", THE JOURNAL OF ORGANIC CHEMISTRY, vol. 71, no. 1, 1 January 2006 (2006-01-01), pages 185-193, XP055320701, US ISSN: 0022-3263, DOI: 10.1021/jo051832o -----	1-48
A	DANIELLE R. SOENEN ET AL: "Synthesis and Inverse Electron Demand Diels-Alder Reactions of 3,6-Bis(3,4-dimethoxybenzoyl)-1,2,4,5-tetrazine", THE JOURNAL OF ORGANIC CHEMISTRY, vol. 68, no. 9, 1 May 2003 (2003-05-01), pages 3593-3598, XP055320704, US ISSN: 0022-3263, DOI: 10.1021/jo020713v -----	1-48
A	Bruce Rickborn: "The Retro-Diels-Alder Reaction Part II. Dienophiles with One or More Heteroatom - Organic Reactions - Rickborn - Wiley Online Library", 15 April 2004 (2004-04-15), XP055320671, Retrieved from the Internet: URL:http://onlinelibrary.wiley.com/doi/10.1002/0471264180.or053.02/abstract [retrieved on 2016-11-17] -----	1-48
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A	WO 2012/156920 A1 (KONINKL PHILIPS ELECTRONICS NV [NL]; ROBILLARD MARC STEFAN [NL]; TEN H) 22 November 2012 (2012-11-22) cited in the application claim 1 ----- -/--	1-48

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/052895

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	KEVIN NEUMANN ET AL: "Nanoparticle "switch-on" by tetrazine triggering", CHEMICAL COMMUNICATIONS - CHEMCOM., vol. 52, no. 75, 1 January 2016 (2016-01-01), pages 11223-11226, XP055320517, ISSN: 1359-7345, DOI: 10.1039/C6CC05118A the whole document -----	1-48
X,P	HAOXING WU ET AL: "A Bioorthogonal Near-Infrared Fluorogenic Probe for mRNA Detection", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 138, no. 36, 14 September 2016 (2016-09-14), pages 11429-11432, XP055320712, US ISSN: 0002-7863, DOI: 10.1021/jacs.6b01625 the whole document -----	1-48

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2016/052895

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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