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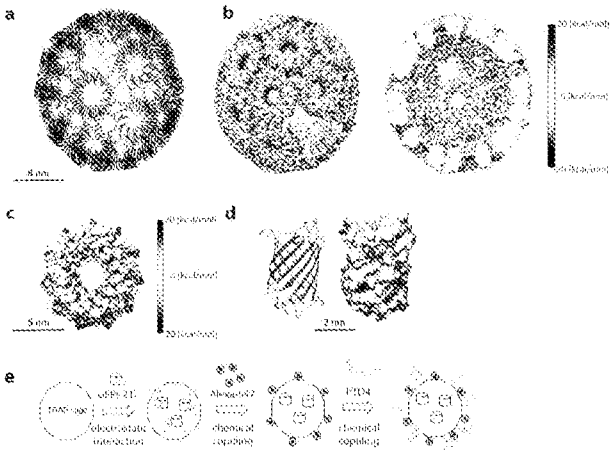
(54) **AN ARTIFICIAL PROTEIN-CAGE COMPRISING ENCAPSULATED THEREIN A GUEST CARGO.**

(57) The present invention provides an artificial TRAP-cage comprising a selected number of TRAP rings and encapsulated therein a guest cargo.



An artificial Protein-cage comprising encapsulated therein a guest cargo

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FIELD OF THE INVENTION

The present invention falls within the biochemistry field. It is related to an artificial protein cage called "TRAP-cage" comprising a selected number of TRAP rings and encapsulated therein a guest cargo.

BACKGROUND

Proteins that assemble into monodisperse cage-like structures are useful molecular containers for diverse applications in biotechnology and medicine. Such protein cages exist in nature, e.g. viral capsids, but can also be designed and constructed in the laboratory.

As such, inventors previously described that a single cysteine mutant of the tryptophan RNA-binding attenuation protein from *Geobacillus stearothermophilus*, TRAP–K35C, can assemble into a hollow spherical structure composed of multiple ring-shape undecameric subunits via reaction with gold nanoparticles[1].The resulted protein cages show an extremely high stability under many harsh conditions, but easily disassemble to the capsomer units by addition of reducing agents.

Although those appealing characteristics of the TRAP cages are ideal to develop an intracellular delivery vehicle, an essential challenge has remained guest packaging.

The object of the invention is to provide a facile and robust method for internal loading of the TRAP-cages with proteins or therapeutics of interest in a stoichiometry controllable manner.

SUMMARY OF THE INVENTION

The subject matter of the invention is an artificial TRAP-cage comprising a selected number of TRAP rings and encapsulated therein a guest cargo.

Preferably the guest cargo is selected from the group comprising a nucleic acid, an enzyme, a therapeutic agent, a small molecule, organic or inorganic nanoparticles, a peptide, a metal, an antigen, an antibody and toxin and fragments thereof of all the foregoing that are of therapeutic value.

Preferably the nucleic acid is selected from the group comprising DNA, RNA, mRNA, siRNA, tRNA and micro-RNA.

Preferably the enzyme is an enzyme associated with an over-expression in a metabolic disorder or disease or an under-expression in a metabolic disorder or disease.

Preferably the enzyme is selected from the group comprising hydrogenase, dehydrogenase, lipase, lyase, ligase, protease, transferase, reductase, recombinase and nuclease acid modification enzyme.

Preferably the therapeutic agent is selected from the group comprising a cancer therapeutic, an anti-infection therapeutic, a vascular disease therapeutic, an immune therapeutic, senolytic and a neurological therapeutic.

Preferably the metal is selected from the group comprising iron, zinc, platinum, copper, sodium, cadmium, lanthanides, gadolinium, technetium, calcium, potassium, chromium, magnesium, molybdenum and salts or complexes thereof.

Preferably toxins are selected from the group comprising a ligand targeted toxin, a protease activated toxin, melittin and a toxin-based suicide gene therapeutic.

Preferably the internal guest cargoes are the same or different from one another.

Preferably the TRAP-cage according to the invention further includes at least one external decoration.

Preferably at least one of the external decorations comprises a cell penetrating agent to promote intracellular delivery of the cage containing an internal guest cargo.

Preferably the cell penetrating agent is PTD4.

Preferably the number of TRAP rings in the TRAP-cage is between 6 to 60.

Preferably the number of TRAP rings in the TRAP-cage is 24.

Preferably opening of the cage is programmable.

Preferably the programmable opening of the cage is dependent on selection of a molecular or atomic cross-linkers which hold the TRAP-rings in place in the TRAP-cage.

Preferably the molecular cross-linker is either (i) a reduction responsive/sensitive linker, whereby the cage opens under reduction conditions; or (ii) a photo-activatable linker whereby the cage opens upon exposure to light.

The subject matter of the invention is also the use of the artificial TRAP-cage according to the invention as a delivery vehicle for intracellular delivery of its internal guest cargo.

The subject matter of the invention is also the use of the artificial TRAP-cage according to the invention as a vaccine.

The subject matter of the invention is also use of the artificial TRAP-cage according to the invention for the treatment of an illness or disease condition selected from the

group comprising cancer, vascular disease, cardiovascular disease, diabetes, infection, auto-immune condition, neurodegenerative disease, cellular senescence disease, arthritis and respiratory disease. LU102571

The subject matter of the invention is also a method of making an artificial TRAP-cage with an encapsulated guest cargo, the method comprising:

(i) obtaining TRAP ring units by expression of the TRAP ring units in a suitable expression system and purification of the said units from the expression system;

(ii) conjugation of the TRAP ring units via at least one free thiol linkage with a molecular cross-linker;

(iii) modification of the TRAP ring units to provide a suitable interior surface environment for capturing a guest cargo;

(iv) formation of the TRAP-cage by self-assembly to provide a cage lumen wherein the guest cargo is encapsulated; and

(v) purification and isolation of the TRAP-cages encapsulating the guest cargo.

Preferably the modification of step (iii) is selected from the group comprising:

(i) super charging the interior surface of the TRAP-cage lumen;

(ii) genetic fusion of the guest cargo to an interior surface of the TRAP-cage lumen;

(iii) SpyCatcher/SpyTag conjugation of the guest cargo to an interior surface of the TRAP-cage lumen; and

(iv) via covalent bond formation in both chemical and enzymatic methods.

Preferably the super charging of step (i) of the interior surface provides either a net positive or net negative charge on the interior surface of the cage lumen.

Preferably the TRAP rings are variants.

Preferably the variant is either TRAP $^{K35C\ E48Q}$ or TRAP $^{K35C\ E48K}$

Preferably the cage formation step of part (iii) for TRAP $^{K35C\ E48Q}$ is performed in sodium bicarbonate buffer at pH 9-11.

Preferably the cage formation step of part (iii) for TRAP $^{K35C\ E48k}$ is performed in sodium bicarbonate buffer at pH 10-10.5.

Preferably the guest cargo can be loaded either pre or post assembly of the TRAP-cage.

Preferably the genetic fusion of the guest cargo to an interior surface of the TRAP-cage lumen of step (ii) is via N-terminus fusion of the guest cargo to an N-terminus of TRAP ^{K35C} which faces into the interior surface of the lumen.

Preferably the SpyCatcher/ SpyTag conjugation of the guest cargo to an interior surface of the TRAP-cage lumen of step (iii) wherein the SpyCatcher is introduced in a loop region of TRAP rings between residues 47 and 48, which faces to the interior when assembled into TRAP-cages and the guest cargo contains a SpyTag.

Preferably enzymatic modification is via peptide ligase selected from the group comprising sortases, asparaginyl, endoproteases, trypsin related enzymes and subtilisin-derived variants and covalent chemical bond formation may include strain promoted alkyne-azide cycloaddition and pseudopeptide bonds.

The subject matter of the invention is also a method of treatment of an individual in need of therapy suffering from a condition selected from the group comprising cancer, vascular disease, cardiovascular disease, diabetes, infection, auto-immune condition, neurodegenerative and neurological disease, cellular senescence diseases, arthritis and respiratory disease, the method comprising administering a therapeutically effective amount of an artificial TRAP-cage bearing one or more internal guest cargoes selected from the group comprising a nucleic acid, an enzyme, a therapeutic agent, a small molecule, organic or inorganic nanoparticles, a peptide, a metal, an antigen, an antibody and toxin and fragments thereof of all the foregoing that are of therapeutic value.

The subject matter of the invention is also a method of vaccinating an individual in need of vaccination from a condition selected from the group comprising cancer, vascular disease, cardiovascular disease, diabetes, infection, auto-immune condition, neurodegenerative and neurological disease, cellular senescence disease, arthritis and respiratory disease, the method comprising administering a therapeutically effective amount of an artificial TRAP-cage bearing one or more internal guest cargo selected from the group comprising a nucleic acid, an enzyme, a therapeutic agent, a small molecule, organic or inorganic nanoparticles, a peptide, a metal, an antigen, an antibody and toxin and fragments thereof of all the foregoing that are of therapeutic value.

Preferably the TRAP-cage therapeutic is administered via intranasal inhalation or injection.

If no cysteine is present in the biomolecule, or they are present but not available for the reaction, -SH group, preferably as a group of cysteine, may be introduced into the biomolecule.

Introduction of cysteine can be carried out by any method known in the art. For example, but not limited to, the introduction of the cysteine is performed by methods known in the art, such as commercial gene synthesis or PCR-based site-directed mutagenesis using modified DNA primers. Above-mentioned methods are known by the persons skilled in the art and ready-to use kits with protocols are available commercially.

-SH moiety may be introduced into the biomolecule also by modification of other amino acids in the biomolecule i.e. by site-directed mutagenesis or by solid phase peptide synthesis.

Reference herein in to "*encapsulation*" within the TRAP-cage is synonymous with enclosed, enveloped, contained or confined with the TRAP-cage.

Reference herein to a "*guest cargo*" refers to the biologic or whatever is encapsulated within the TRAP-cage.

Reference herein to "*TRAP ring*" is synonymous with a TRAP building block, a subunit of the TRAP-cage complex or a TRAP monomer assembly.

"*Unit*", "*subunit*", "*molecule*", "*biomolecule*", "*monomer*" are used alternatively in the description and means one molecule which connects to another molecule for the complex formation.

"*Complex*", "*assembly*", "*aggregate*", are used alternatively in the description and means a superstructure constructed by the reaction between biomolecules. The amount of the units involved in the complex depends of the nature of the biomolecule. More specifically, it depends on the amount of the biomolecule and the amount of -SH groups present in the biomolecule.

Moreover, following abbreviations have been used: TRAP (trp RNA-binding attenuation protein), GFP (green fluorescence protein), PTD4 (protein transduction domain), CPP (cell penetrating peptide), SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), TEM (transmission electron microscopy), DMEM (Dulbecco's Modified Eagle Medium), FBS (foetal bovine serum).

TRAP protein is a suitable biomolecule model for the method of the invention. This is likely due to its high intrinsic stability, toroid shape, lack of native cysteine residues (for easier control of the conjugation process) and availability of a residue that can be

changed to cysteines with the resulting cysteine being in a suitable chemical and spatial environment suitable for proper bond formation.

Nevertheless, a person skilled in the art would easily adapt the reaction conditions for other biomolecular monomers. Any biomolecular monomer that has free thiol(s) group(s) and/or its structure allows modification by introducing thiol group may be suitable for the method of conjugation of the biomolecules according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

Transport of molecular cargoes to cells is desirable for a range of applications including delivery of drugs, genetic material or enzymes. A number of nanoparticles have been employed to achieve this including liposomes, virus-like particles, non-viral protein cages, DNA origami cages and inorganic nanoparticles, each with their own advantages and disadvantages. Protein cages are a promising approach as demonstrated by viruses in nature which are able to deliver genetic material to cells, often with high efficiency and specificity.

Artificial cages are constructed by proteins which do not naturally form cage structures and in which interactions between constituent proteins may be modified to promote their assembly. The advantage of using such an approach is that the resulting cages can be given properties and capabilities that may not be available or feasible in naturally occurring forms. To date a number of artificial protein cages have been produced including tandem fusions of proteins with 2- and 3-fold rotational symmetries able to form a 12-subunit tetrahedral cage, a nanocube structure of 24 subunits with octahedral symmetry, a 60-subunit icosahedral cage structure that self-assembles from trimeric protein building blocks, and co-assembling two-component 120-subunit icosahedral protein complexes comparable to those of small viral capsids as well as designed peptides able to form networks that close to form cages. Several examples exist where artificial protein cages have been filled with various cargoes including siRNA, mRNA[2] and fluorescent dyes. However, only a handful of cases have demonstrated delivery of cargo to cells by artificial cages. To the best of our knowledge, delivery of protein/therapeutic cargoes to cells mediated by artificial protein cages (as opposed to natural cages) has not previously been demonstrated.

We previously produced an artificial protein cage using a building block consisting of the naturally occurring ring-shaped protein, TRAP (trp RNA-binding attenuation protein) referred to as TRAP-cage (**Fig. 1a**)[1]. In nature, TRAP is involved in control of tryptophan synthesis and has been well characterised structurally and biochemically. It has also been used as a versatile building block in bionanoscience. TRAP-cage

consists of 24 TRAP rings forming an approximately 22 nm diameter, 2.2 MDa hollow sphere with a lumen roughly 16 nm in diameter. Each TRAP ring in the cage is bound to 5 TRAP ring neighbours and the structure contains 6 square holes approximately 4 nm in diameter. Unusually, compared to other natural and most artificial cages, the ring subunits in the cage are held together not by a network of protein-protein interactions. Instead, single gold(I) ions bridge opposing sulphurs of the cysteine residues between rings in proteins where naturally occurring lysine at position 35 is replaced with cysteine. The cysteines of ten of the 11 monomers of each ring in the cage are bridged in this way with an eleventh remaining unbridged and available to react, e.g. with maleimide-labelled dyes.

TRAP-cage is extremely stable, able to survive temperatures of 95 °C for at least 3 hours, and high levels of denaturing agents such as urea. Despite this high stability TRAP-cage breaks apart readily in the presence of low concentrations of reducing agents including the cellular reducing agent glutathione. This feature raises the prospect that the TRAP-cage may have utility as a system for delivering cargo to cells, as it can be expected to retain its structure, protecting cargo until entering cells where intracellular reducing agents will result in disassembly and subsequent cargo release.

We have shown that TRAP-cage can be deliberately filled with protein cargo, and we use a negatively supercharged variant of green fluorescent protein, GFP(-21), as an exemplar molecule. We also show that TRAP-cage can be used to deliver such cargoes to the interiors of human cells. This cell-penetration is itself controllable as it only occurs if the surface of TRAP-cage is modified, e.g. by cell-penetrating peptide. The results are a first step towards development of TRAP-cage as a potentially useful tool for delivering medically relevant cargoes to cells and more generally demonstrates the potential for artificial protein-cage systems as therapeutic agents.

Here we show that TRAP-cage can be used to deliberately encapsulate a protein cargo and deliver it to cell interiors. TRAP- cages employed either in unmodified form or externally decorated, showed no significant effects on cell viability.

In this case, filling with cargo was achieved using our previously developed TRAP-cage[1] having positively charged patches on its interior, to capture negatively supercharged GFP electrostatically through diffusion into the cage. Attempts to deliver filled cages to cells showed no evidence of penetration of TRAP-cages into cells if they were undecorated. In contrast, attachment of cell penetrating peptide (CPP) PTD4 to the exterior of TRAP-cages resulted in significant penetration into cell interiors.

A small number of previous works on artificial protein cage-mediated delivery of cargo to cells have demonstrated success for non-protein cargoes. Notably, it has been shown that an artificial protein cage loaded with siRNA can be taken up by different mammalian cells and release its cargo to induce RNAi and knockdown of target gene expression[3]. In this case, the high gene silencing efficiency together with low toxic effects indicated that a protein cage carrier has potential as a therapeutic delivery system. Encapsulation of protein cargoes within artificial protein cages has previously been demonstrated. However, these cages were not shown to be able to directly deliver their cargo to cells, instead multiple copies of the cages were themselves used as cargoes within lipid envelopes made in cells and purified as enveloped protein nanocages' (EPNs) where the lipid envelope was derived from the host cell membrane. The EPNs were able to deliver the cages meaning that entry to cells was achieved by the enveloping, host-derived membrane, not the protein cage.

Given the overall high stability of TRAP-cage but its proven ability to disassemble in the presence of cellular reducing agents[1] it would be interesting to know if cages readily break apart once inside cells. The change in relative signal strengths of TRAP-cage associated Alexa-647 versus GFP once in the cell is suggestive of intracellular break-up of the cage and release of the cargo. A possible explanation is that when Alexa-647 and GFP are in close proximity to each other due to association with TRAP-cage, the GFP fluorescence may be decreased due to a quenching effect from the dye. Once GFP is released by TRAP-cage disassembly, average GFP to Alexa-647 distances become larger, resulting in an increase in detected GFP fluorescence. This possibility is supported by the observation that the signal from intracellular GFP is visibly brighter when it is delivered using TRAP-cage lacking Alexa-647 (Figure 10).

Overall, the work presented herein offer a first demonstration of protein delivery to cells mediated by artificial protein cages. The cargo-filling efficiency demonstrated was quite low and this could be addressed by modifying TRAP-cage further such that it carries a higher density of positive charge within the cage interior. Alternatively, different methods of cargo capture (such as covalent attachment) could be explored, as described for other protein cages.[4,5] Additionally we anticipate further modification of TRAP-cage both to increase targeting specificity and to extend the range and usefulness of encapsulated cargo. Finally, future studies will be required to pinpoint and track both the precise intracellular location of TRAP-cages and their quaternary state.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. TRAP-cage protein. (**a**) Structure of TRAP-cage (PDB:6RVV) with each TRAP-ring shown a different colour. Gold atoms are shown as yellow spheres. (**b**) Surface representation of TRAP-cage exterior (left) and interior (right) coloured by charge distribution. (**c**) Surface view of a single TRAP-ring with the face that points into the interior cavity shown, coloured according to charge. (**d**) Negatively supercharged GFP(-21) shown in cartoon representation (left) and surface view coloured according to charge (right). (**e**) Scheme of TRAP-cage encapsulation with GFP(-21) and external modifications with Alexa-647 dye and PTD4 peptide.

Fig. 2. Filling and decoration of TRAP-cage. (**a**) Native PAGE gels showing purified TRAP-cage incubated with His-tagged GFP(-21) after passing through a Ni-NTA column in the absence (- TCEP) or presence (+ TCEP) of TCEP. Lane 1: GFP(-21) positive control; 2: molecular weight marke for native PAGE; 3: empty TRAP-cage; 4: input (TRAP-cage with GFP(-21)); 5 and 8: flow-through; 6 and 9: wash; 7 and 10: elution. Collected fractions were stained for protein (left) or analysed by fluorescence detection (right, exct. 488 nm). (**b**) Collected fractions were subjected to SDS-PAGE followed by Western blot with anti-GFP detection. Lane 1: GFP(-21) positive control; 2: molecular weight marker for SDS-PAGE; 3: empty TRAP-cage; 4: input (TRAP-cage with GFP); 5 and 8: flow-through; 6 and 9: wash; 7 and 10: elution. (**c**) Native PAGE gels showing encapsulation of GFP(-21) by unmodified TRAP-cage or TRAP-cage externally modified by Alexa-647 and PTD4. Lane 1: TRAP-cage with GFP(-21); 2: TRAP-cage with GFP(-21) decorated with Alexa-647; 3: TRAP-cage with GFP(-21) decorated with Alexa-647 and PTD4; 4: molecular weight marker for native PAGE. Gels were stained for protein (upper panel) and analysed by fluorescence detection of GFP (middle panel, exct. 488 nm) and Alexa-647 (bottom panel, exct. 647). (**d**) Negative stain transmission electron microscopy of TRAP-cage with GFP(-21) (left panel); TRAP-cage with GFP(-21) decorated with Alexa-647 (middle panel); TRAP-cage with GFP(-21) decorated with Alexa-647 and PTD4 (right panel).

Fig. 3. Delivery of TRAP-cage carrying GFP(-21) to MCF-7 cells. (**a**) Representative flow cytometry dot plots of MCF-7 cells after 4 h treatment with Alexa-647 labelled TRAP-cage carrying GFP(-21) (denoted as (TC+GFP) + Alexa-647) and TRAP-cage with GFP(-21) labeled with Alexa-647 and PTD4 peptide (denoted as (TC+GFP) + Alexa-647 + PTD4) for 15 min, 2 h and 4 h. The x-axis and the y-axis show the fluorescent intensities of GFP and Alexa-647, respectively. Untreated cells were used as the negative control. (**b**) Representative red and green fluorescence overlay histogram plot of MCF-7 cells from the same experiment. (**c**) Median fluorescence

intensity of Alexa-647 and GFP positive cells treated with TRAP-cage carrying GFP and decorated with Alexa-647 or decorated with both Alexa-647 and PTD4 after 15 min, 2 h and 4 h incubations. Data are normalized to untreated cells and based on three independent experiments. Controls: 1: untreated cells; 2: cells incubated with (TC+GFP) + Alexa-647. (**d**) Confocal microscopy images of untreated cells (control cells, upper row), cells incubated with TRAP-cage filled with GFP(-21) and labeled with Alexa-647 only (middle row): cells incubated with TRAP-cage filled with GFP(-21) and labeled with Alexa-647 and PTD4 (bottom row). Actin filaments were stained with phalloidin conjugated to Alexa-568 and nuclei were stained with DAPI. Green channel – GFP; red channel – Alexa-647; blue channel – DAPI; grey channel – Alexa-568; (scale bar: 10 μM).

Fig. 4. Tracking TRAP-cage and GFP(-21) in MCF-7 cells. Confocal microscopy merged images of cells incubated with TRAP-cage carrying GFP(-21) decorated with Alexa-647 and PTD4 and fixed at different time points. Actin was stained with phalloidin conjugated to Alexa-568 whereas DAPI was used for nuclear staining; (scale bar: 10 μM). Rectangular images beneath each main image are representative orthogonal views in the yz axis. (**a**) - images with red channel maximal projection; (**b**) - images with green channel maximal projection.

Fig. 5. Estimating the number of His-tagged GFP(-21) molecules in the TRAP-cage. (**a**) Standard curve obtained from fluorescence measurements of GFP(-21) protein, with the concentration range from 0 - 100 nM. Fitted with equation: $y = 0.0258x + 4.4$; $R^2 = 0.9786$. (**b**) Western blot used for band densitometry analysis. Lanes 1-4: GFP(-21); lane 5: TRAP-cage loaded with GFP(-21) (denoted as (TC+GFP)).

Fig. 6. External decoration of TRAP-cage with GFP(-21) (**a**) RP-HPLC chromatogram showing purified PTD4 peptide used to decorate TRAP-cage filled with GFP(-21). (**b**) Native PAGE gels showing TRAP-cage carrying GFP(-21) after titration of Alexa-647 in the conjugation reaction. Gels were analysed by fluorescence detection of Alexa-647 (left panel, exct. 647) and GFP (middle panel, exct. 488 nm) and stained for proteins (right panel). Arrows show optimal decoration conditions used in further experiments. (**c**) SDS-PAGE gel comparing TRAP-cages carrying GFP(-21) either with no decoration, decorated with Alexa-647 or decorated with both Alexa-647 and PTD4. Left: detection at 488 nm; middle: detection at 647 nm; right: Western blot of the same samples detected with anti-GFP antibody. Lanes: 1: molecular weight marker for SDS-PAGE electrophoresis; 2: TRAP-cage with GFP(-21); 3: TRAP-cage with GFP(-21) decorated with Alexa-647; 4: TRAP-cage with GFP(-21) decorated with Alexa-647 and PTD4; 5: GFP(-21) - positive control.

Fig. 7. TRAP-cage stability in culture medium and cell viability test. (**a**) Native PAGE gels showing TRAP-cage stability in DMEM culture medium without and with FBS presence during 18 h incubation. (**b**) Cell viability of MCF-7 and HeLa cells after 4 h exposure to empty TRAP-cage, TRAP-cage loaded with GFP(-21) and TRAP-cage with GFP(-21) decorated with Alexa-647 and PTD4. M = molecular weight marker for native electrophoresis; TC: empty TRAP-cage; (TC+GFP): TRAP-cage filled with GFP(-21); (TC+GFP) + Alexa-647 + PTD4: TRAP-cage with GFP(-21) and decorated with Alexa-647 and PTD4.

Fig. 8. Delivery of TRAP-cage with GFP(-21) to HeLa cells. (**a**) Representative flow cytometry dot plots of HeLa cells after treatment with Alexa-647 labeled TRAP-cage with GFP(-21) for 4 h (denoted as (TC+GFP) + Alexa-647) and Alexa-647 labeled TRAP-cage with GFP(-21) and PTD4 (denoted as (TC+GFP) + Alexa-647 + PTD4) for 15 min, 2 h and 4 h. The x-axis and the y-axis show the fluorescent intensities of GFP and Alexa-647 respectively. Untreated cells were used as the negative control. (**b**) Representative red and green fluorescence overlay histogram plot of the HeLa cells from the same experiment. (**c**) Median fluorescence intensity of Alexa-647 and GFP positive cells treated with (TC+GFP) + Alexa-647 and (TC+GFP) + Alexa-647 + PTD4 after 15 min, 2 h and 4 h incubation. Data are normalized to untreated cells and based on three independent experiments. Controls: 1: untreated cells; 2: cells incubated with (TC+GFP) + Alexa-647 (**d**). Confocal microscopy images of untreated cells (control cells) (upper row), cells incubated with (TC+GFP) labeled with Alexa-647 only (middle row), cells incubated with TRAP-cage filled with GFP(-21) and labeled with Alexa-647 and PTD4 (bottom row). Actin filaments were stained with phalloidin conjugated to Alexa-568 and nuclei were stained with DAPI. Green channel – GFP; red channel – Alexa-647; blue channel – DAPI; grey channel – Alexa-568; (scale bar: 10 μM).

Fig. 9. Tracking TRAP-cage and GFP in HeLa cells. Confocal microscopy merged images of cells incubated with TRAP-cage with GFP(-21) labeled with Alexa-647 and PTD4 and fixed in different time points. Actin was stained with phalloidin conjugated to Alexa-568 whereas DAPI was used for nuclear staining; (scale bar: 10 μM). Rectangular images are representative orthogonal views in the yz axis. (**a**) - images with red channel maximal projection; (**b**) - images with green channel maximal projection.

Fig. 10. Influence of Alexa-647 of GFP(-21) fluorescence. (**a**) Cells were exposed to (TC+GFP) labeled with Alexa-647 and PTD4 (upper row) or (TC+GFP) labeled with PTD4 only (lower row). Actin filaments were stained with phalloidin conjugated to Alexa-568 and nuclei were stained with DAPI. Green channel – GFP; red channel –

Alexa-647; blue channel – DAPI; grey channel – Alexa-568; (scale bar: 10 μM). (**b**) Mean GFP fluorescence intensity registered from three different fields of view for samples where cells were exposed to (TC+GFP) labeled with Alexa-647 and PTD4 or (TC+GFP) labeled with PTD4 only. The fluorescence intensity was quantified with ImageJ, considering background intensity subtraction. (**c**) Mean fluorescence of GFP(-21) encapsulated in the undecorated and fully decorated TRAP cage, measured in solution.

EXAMPLES

Techniques employed in the realisation of the invention

Electron microscopy

TRAP-cage filled with GFP(-21), TRAP-cage filled with GFP(-21) and labelled with Alexa-647, and TRAP-cage filled with GFP(-21) and fully decorated were imaged using a transmission electron microscope. Samples were typically diluted to a final protein concentration of 0.025 mg/ml, centrifuged at 10 000 g, 5 min, at room temperature and the supernatant applied onto hydrophilized carbon-coated copper grids (STEM Co.). Sample were then negatively stained with 3% phosphotungstic acid, pH 8, and visualized using a JEOL JEM-2100 instrument operated at 80 kV.

Flow cytometry

For TRAP-cage internalization experiments, MCF-7 and HeLa cells were seeded into 12-well plates (VWR) in 800 μl of DMEM medium with 10% FBS at a density of 2.5 x 10^5 per well and cultured for a further 16 h prior to the experiments. Cells were then incubated with 50 μg (6 nM) of TRAP-cage filled with cargo, labelled with Alexa-647 only or decorated with Alexa-647 and PTD4 peptide in 50 mM HEPES with 150 mM NaCl pH 7.5 supplemented with 10% FBS for 15 min, 2 h and 4 h. After the incubation, cells were washed three times for 5 min with phosphate buffered saline (PBS) (EURx), harvested with trypsin (1 mg/ml) and centrifuged at 150 *g* for 5 min. Subsequently, cells were washed thrice in PBS by centrifugation (150 *g* for 3 min) and re-suspended in PBS. Cells were run in Navios flow cytometer (Beckman Coulter) and the fluorescence of 12000 cells was collected per each sample. Untreated cells and cells treated with TRAP-cage filled with cargo and labelled with Alexa-647 only were used as negative controls. Obtained data for three independent experiments were analyzed with Kaluza software (Beckman Coulter). The percentage of Alexa-647/GFP positive cells and median fluorescence intensity was determined for each sample.

Laser Scanning Confocal Microscopy

For fluorescent laser scanning confocal microscope observations, cells were grown on 15-mm glass cover slips plated into 12-well plates (2.5 x 10^5 per well in 800 µl DMEM medium with 10% FBS) and further stimulated as described above for flow cytometry experiments. Next, cells were washed with PBS (3 times for 5 min), fixed with 4% paraformaldehyde solution (15 min, at room temperature) and permeabilized with 0.5% Triton-X100 in PBS (7 min, at room temperature). Actin filaments were stained with phalloidin conjugated to Alexa-568 in PBS (1:300, Thermo Fisher Scientific, 1.5 h, at room temperature). Cover slips were then mounted on slides using Prolong Diamond medium with DAPI (Thermo Fisher Scientific). Fluorescent images were acquired under Axio Observer.Z1 inverted microscope (Carl Zeiss, Jena, Germany), equipped with the LSM 880 confocal module with 63x oil immersion objective. Images were processed using ImageJ 1.47v (National Institute of Health).

Example 1. Filling of TRAP-cage.

To fill TRAP-cage we took advantage of the fact that the only significant patch of positive charge on the surface of the TRAP ring lies on the face lining the interior of the cage **Fig. 1a-c 1b, c**). In principle this could allow capture of negatively charged cargoes via electrostatic interaction as has been demonstrated for other protein cages (e.g.[6]) The fact that the constituent TRAP rings do not assemble into TRAP-cage until the addition of gold(I)[1] means that protein cargoes below approximately 4 nm have two possible routes to encapsulation – they may bind to TRAP rings prior to assembly or they may be added after TRAP-cage formation and allowed to diffuse into the cage through the 4-fold holes. We chose negatively supercharged GFP(-21) as a model cargo (**Fig 1d**). This cylindrically shaped protein has a diameter of approximately 2.4 nm and is therefore expected to be able to diffuse into the assembled TRAP-cage (**Fig 1e**). His-tagged GFP(-21) was mixed with TRAP-cages and incubated overnight, followed by size exclusion chromatography purification for removal of remaining free GFP(-21). It was found that the two proteins associated as shown by co-migration of fluorescence signals on native gels (**Fig 2a**). To verify whether His-tagged GFP(-21) is inside the TRAP-cage and not bound to its exterior, we conducted a pull down assay using Ni-NTA affinity chromatography, followed by Western blot analysis. The observation that the GFP(-21) associated with TRAP-cage did not bind to the Ni-NTA column suggested successful encapsulation, making the His-tag inaccessible. This was further supported by a pull down assay which showed that the associated GFP(-21) was only available to interact with a Ni-NTA column after the cage was dissociated by the addition of reducing agent (**Fig 2b**). These results strongly suggest encapsulation of GFP in TRAP-cage in either full of partial modes (partial

encapsulation being the case where the GFP "plugs" the holes in TRAP-cage with the His-tags pointing to the interior). The number of GFP(-21) per cage was approximately 0.3, comparable to that found in a number of other filled protein cages though some have shown considerably greater numbers of cargoes.

Production and purification of TRAP-cage filled with GFP(-21)

TRAP-cage production and purification was performed as described previously.[1] For relevant plasmid and amino acid sequence information see Table 1. Supercharged (-21) His-tagged GFP protein was expressed from pET28a encoding the GFP gene and produced in BL21(DE3) cells. The protein was purified using Ni-NTA. Briefly, cells were lysed by sonication at 4 °C in 50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, in presence of protease inhibitors (Thermo Fisher Scientific), and lysates were centrifuged at 20 000 *g* for 0.5 h at 4 °C. The supernatant was incubated with agarose beads coupled with Ni^{2+}-bound nitrilotriacetic acid (His-Pur Ni-NTA, Thermo Fisher Scientific) preequilibrated in 50 mM Tris, pH 7.9, 150 mM NaCl, 20 mM imidazole (Buffer A). After three washes of the resin (with Buffer A) the protein was eluted with 50 mM Tris, pH 7.9, 150 mM NaCl, 300 mM imidazole (Buffer B). Fractions containing His-tagged GFP(-21) were pooled and subjected to size exclusion chromatography on a HiLoad 26/600 Superdex 200 pg column (GE Healthcare) in 50 mM Tris-HCl, pH 7.9, 150 mM NaCl at room temperature. Protein concentrations were measured using a Nanodrop spectrophotometer using a wavelength of 280 nm.

GFP encapsulation was conducted by mixing equal volumes of 100 µM negatively supercharged (-21) His-tagged GFP with 1 µM pre-formed TRAP-cage incubating overnight in 50 mM Tris, 150 mM NaCl, (pH 7.9). Purification of TRAP loaded with GFP was carried out by size exclusion chromatography using a Superose 6 Increase 10/300 column (GE Healthcare) in 50 mM HEPES, pH 7.5, 150 mM NaCl. Fractions containing TRAP-cage were collected and analyzed by native PAGE using 3-12% native Bis-Tris gels (Life Technologies) followed by fluorescence detection using a Chemidoc detector (BioRad) with excitation at 488 nm.

Estimating the number of His-tagged GFP(-21) molecules in the TRAP-cage

Two methods were used for estimating the loading of GFP(-21):

1. Based on detection of GFP fluorescence in TRAP-cage filled with cargo. A GFP(-21) standard curve was prepared in the concentration range of 0-100 nM. The fluorescence spectra were acquired at 26 °C using a RF-6000 Shimadzu® Spectro Fluorophotometer with a fixed excitation wavelength at 488 nm and emission wavelength range of 495–550 nm, with an interval of 1.0 nm for λ_{em}, scan speed 6000

nm min $^{-1}$, λ_{ex} bandwidth 5 nm and λ_{em} bandwidth 5 nm. The fluorescence at emission maximum λ_{em} 510 nm was used for calculation. TRAP protein concentration was determined from absorbance at 280 nm. A TRAP-cage : GFP(-21) stoichiometry of 1: 0.28±0.07 was obtained (**Fig. 5a**). LU102571

2. Densitometry analysis. Briefly, a series of His-tagged GFP(-21) dilutions (0.4 ng; 0.8 ng; 4 ng; 8 ng as measured by Nanodrop at wavelength 280 nm) and TRAP-cage filled with cargo, sample (2 µg as measured by Nanodrop at wavelength 280 nm) were separated by SDS-PAGE and subjected to Western blotting (**Fig. 5b**).The signal from His-tagged GFP(-21) protein was detected with anti-GFP antibody and secondary HRP-conjugated antibody in a chemiluminescence detector (Chemidoc, BioRad). Densitometry analysis using ImageLab (BioRad) software of the resulting blot showed that 0.6 ng of His-tagged GFP(-21) was present in 2 µg of TRAP-cage filled with cargo. The densitometry analysis yielded a TRAP-cage : GFP(-21) stoichiometry of approx. 1 : 0.4.

Ni-NTA "pull down"

Samples of purified TRAP-cage filled with His-tagged GFP(-21) protein were divided into two portions and incubated under reducing (1 mM TCEP) or non-reducing (no TCEP) conditions. Next, samples were passed through a Ni-NTA resin (Thermo Fisher Scientific) under gravitational flow in which 100 µl of each sample was introduced onto 50 µl of the resin equilibrated with Buffer A. Three samples were collected: (i) flow through, (ii) wash with Buffer A and (iii) elution with Buffer B. Samples were analyzed by native PAGE, followed by fluorescence detection (excitation at 488 nm, Chemidoc, BioRad) and Western blot. For the SDS-PAGE and Western blot samples collected from the Ni-NTA pull down assay were denatured by addition of TCEP (final concentration 0.1 mM) and boiling for 15 min followed by separation via Tris/Glycine gel electrophoresis. The gel was subjected to electrotransfer (2 h, 90 V) in 25 mM Tris, 192 mM glycine, 20% methanol buffer onto an activated PVDF membrane. The membrane was blocked with 5% skimmed milk in Tris-buffered saline supplemented with 0.05% of Tween 20 (TBS-T), followed by 1.5 h incubation with mouse monoclonal anti-GFP antibody (1:2500; St. John's Laboratories, UK) and anti-mouse (1:5000, Thermo Fisher Scientific) secondary antibody conjugated with horse radish peroxidase. The signal was developed using a Pierce ECL Blotting Substrate (Thermo Fisher Scientific) and visualized in a BioRad Chemidoc detector.

Table 1. Plasmid information and amino acid sequences

Sequence ID	Plasmid name	Plasmid	Gene	Amino acid sequence
SEQ ID NO: 1	pET21b TRAP-K35C-E48Q-H	pET21b	TRAP-K35C-E48Q	MYTNSDFVVIKALEDGVNVIG LTRGADTRFHHSECLDKGEVL IAQFTQHTSAIKVRGKAYIQTR HGVIESEGKK
SEQ ID NO: 2	pET21b TRAP-K35C-E48K-H	pET21b	TRAP-K35C-E48K	MYTNSDFVVIKALEDGVNVIG LTRGADTRFHHSECLDKGEVL IAQFTKHTSAIKVRGKAYIQTR HGVIESEGKK
SEQ ID NO: 3	pET21b TRAP-K35C	pET21b	TRAP-K35C	MYTNSDFVVIKALEDGVNVIG LTRGADTRFHHSECLDKGEVL IAQFTEHTSAIKVRGKAYIQTR HGVIESEGKK
SEQ ID NO: 4	pET21b TRAP-K35C R64S	pET21b	TRAP-K35C R64S	MYTNSDFVVIKALEDGVNVIG LTRGADTRFHHSECLDKGEVL IAQFTEHTSAIKVRGKAYIQTS HGVIESEGKK
SEQ ID NO: 5	pET28a GFP(-21)	pET28a	GFP(-21)	HHHHHGSACELMVSKGXELXX GVVPILVELDGDVNGHEFSV RGEGEGDATEGELTLKFICTT GKLPVPWPTLVTTLTYGVQCF SRYPDHMKQHDFFKSAMPEG YVQERTISFKDDGTYKTRA EVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNFNSHDVYI TADKQENGIKAEFEIRHNVED GSVQLADHYQQNTPIGDGPV LLPDDHYLSTESALSKDPNEK RDHMVLLEFVTAAGITHGM D ELYK
Sequence ID	**Peptide**			**Amino acid sequence**
SEQ ID NO: 6	PTD4			Ac-YARAAARQARAG

Example 2. Decoration of TRAP-cage with fluorescent dye and with cell-penetrating peptide labelling.

We aimed to modify the TRAP-cage in order to promote its cell entry. We choose PTD4 (YARAAARQARA, SEQ. ID No. 7) – an optimised TAT-based cell-penetrating peptide that shows significantly improved ability to penetrate cell membranes, being more amphipathic with a reduced number of arginines and increased α-helical content.[7] A number of works have shown that coating nanoparticles with PTD4 or similar promotes cell penetration (e.g.[8]). We attached the PTD4 derivative, Ac-YARAAARQARAG, to the amino groups on surface exposed lysines of TRAP-cages. There are three such surface exposed lysines per monomer on TRAP-cage, potentially allowing 792

peptides to be attached per cage. Acetylation of the N-terminal amino group eliminates the possibility of cross-reaction of those amino groups with activated carboxyl moieties that are intended to react with available amino groups of TRAP protein. Additionally, the extended C-terminal glycine residue serves as a flexible linker and as it is not a chiral amino acid, abolishes the chance of racemization during carboxyl activation. The peptide was synthesized using solid-phase methodology and purified by reverse-phase high-performance liquid chromatography (**Figure 6a**). In optimised reactions we observed an increase in the apparent molecular weight of TRAP-cage after reaction with PTD4 (**Figure 6c**) as visualised by native PAGE.

In order to be able to track TRAP-cage independently from its cargo we labelled it with Alexa-647 fluorescent dye. For this we cross-linked the maleimide group on the dye with the 24 available cysteines lining the six 4-nm holes of TRAP-cage that are not involved in ring-ring interactions. By titration we established the optimal amount of Alexa-647 (which was equal to the number of TRAP cysteine groups) to be added, where the TRAP-cage is readily labelled and no free dye is present in the sample. This was assessed by native PAGE combined with fluorescent measurements to detect both GFP(-21) and Alexa-647 (**Figure 6b**). Although the cargo GFP contains 3 cysteine residues, control reactions showed no detectable labelling of GFP with Alexa-647 (**Figure 6c**). Negative stain transmission electron microscopy (TEM) confirmed that the modified TRAP-cages retained their characteristic shape (**Figure 6d**).

PTD4 peptide synthesis

PTD4 peptide derivative (Ac-YARAAARQARAG, for simplicity called PTD4 in the text) was synthesized at 0.1 mmol scale using a Liberty Blue automated microwaved synthesizer (CEM, USA), according to the Fmoc-based solid phase peptide synthesis methodology. Fmoc-Gly-Wang resin (100-200 mesh, substitution 0.70 mmol/g, Novabiochem, Germany) was swelled overnight with dichloromethane (DCM)/dimethylformamide (DMF) (1:1). Fmoc-deprotection was performed with 25% morpholine in DMF for 5 min at 85 °C. Coupling reactions were performed as per recommended manufacturer's protocol using DIC/oxyma activators with a fivefold excess of Fmoc-protected amino acid derivatives for 5 min at 85 °C. Double coupling was applied for all Fmoc-Arg (Pbf) coupling. N-terminal acetylation was performed on resin with 10% acetic anhydride in DMF at 60 °C. Cleavage from the resin and side chains deprotection were achieved by treatment with TFA/Triisopropylsilane (TIS)//water (94:3:3) for 4 h with vigorous shaking at 30 °C. The resin was filtrated and TFA was evaporated under a mild nitrogen stream. The crude peptide was precipitated by addition of cold diethyl ether, followed by centrifugation (3000 rpm, 10 min). The

residue was washed with cold ether (2x) and ethyl acetate (2x). Precipitated crude peptide was dried *in vacuo* overnight. Crude peptide was dissolved in 8 M urea and purified on an Agilent 1260 RP-HPLC using semi-preparative C18 (10x150 mm) column (Cosmosil, Nacalai tesque). Collected peptide-containing fractions were lyophilized. Purified peptide was analyzed on an analytical C18 column (Zorbax SB-C18 5mm 4.6x150 mm, Agilent) in a linear gradient of 0 – 20% of acetonitrile with 0.1% TFA for 30 min at flow rate 1.0 ml/min. Peak signals were detected at 220 and 280 nm (**Fig. 6a**).

TRAP-cage labeling with Alexa-647 and decoration with cell-penetrating peptide

Alexa Fluor-647 C2 maleimide fluorescent dye (Alexa-647, Thermo Fisher Scientific) and cell-penetrating PTD4 peptide were conjugated to the TRAP-cage filled with GFP via a crosslinking reactions with cysteines and lysines present in the TRAP protein.

To achieve fluorescent labelling, TRAP-cage carrying GFP (300 µl, 16 nM) was mixed with a Alexa-647 C2 maleimide dye (50 µl, 1 µM), the reaction was conducted in 50 mM HEPES with 150 mM NaCl pH 7.5 for 2.5 h at room temperature with continuous stirring at 450 rpm. The optimal interaction ratio of maleimide-conjugated Alexa-647 to TRAP-cage was assessed by titration (**Fig. 6b**). Briefly, aliquots of TRAP-cage loaded with GFP(-21) (11.36 nM) were mixed with maleimide-conjugated Alexa-647 ranging from 0.1 µM to 100 µM. Samples were then separated by native gel electrophoresis and visualized by fluorescence detection in a Chemidoc, with excitation at 647 nm. Reactions where no free Alexa-647 is present in the sample and no GFP interference with the Alexa-647 signal is observed, were considered as optimal decoration conditions and used in further experiments.

Additionally, to rule out a possibility of direct GFP labeling by Alexa-647, TRAP-cage loaded with GFP(-21) with and without Alexa-647 labelling were subjected to denaturing gel separation and Western blotting followed by detection with anti-GFP antibody. No band shift from potential interaction of GFP with Alexa-647 dye was observed (**Fig. 6c**).

For the cell-penetrating peptide decoration, PTD4 peptide (50 µl, 0.5 mM) was mixed with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 10 µl, 83 mM) and *N*-hydroxysuccinimide (NHS, 10 µl, 435 mM), all reagents dissolved in ddH_2O. Subsequently, the excess of activated PTD4 peptides were added to TRAP-cage filled with GFP(-21) and labelled with Alexa-647 and incubated for next 2.5h at room temperature, with continuous stirring at 450 rpm. The reaction was stopped by addition of 5 µl of 200 mM Tris-HCl pH 7.5. The conjugation efficiency was verified by

native PAGE and fluorescent gel imaging. A change in molar weight of the decorated TRAP-cage results in a band shift observed in native PAGE (**Figure 6c**).

Example 3. Stability of TRAP-cage and effect on cell viability.

Before embarking on cell delivery tests, we firstly assessed whether TRAP-cage was structurally stable, i.e. did not disassemble under cell culture conditions. Stability was checked at 37 °C, 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) without or with foetal bovine serum (FBS) at various concentrations. The results showed that the cage structure is stable in the DMEM culture medium within 18 h incubation at 37 °C, 5% CO_2 (**Figure 7a**).

In order to determine the effect of TRAP-cage on cell viability alamarBlue assays were carried out. This test is based on the natural ability of viable cells to convert resazurin, a blue and nonfluorescent compound, into resofurin; a red and fluorescent molecule by mitochondrial and other reducing enzymes.[9] Human cancer cell lines MCF-7 and HeLa were incubated in the presence of a TRAP-cage, TRAP-cage filled with GFP(-21) and decorated with Alexa-647 and PTD4 peptide. The number of cells, TRAP-cage dose and stimulation time used in cell viability tests correspond to the conditions under which the internalization of the TRAP-cage experiments were performed. Untreated cells were used as a control. The data showed that both unmodified TRAP-cage and TRAP-cage filled with GFP(-21) and decorated with Alexa-647 and PTD4 do not significantly affect the viability of MCF-7 and HeLa cells for at least 4 h of incubation (**Figure 7b**).

Cell culture and cytotoxicity assessment of the TRAP-cage

HeLa and MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% FBS (EURx), 100 µg/ml streptomycin, 100 IU/ml penicillin (Gibco). The culture was maintained at 37 °C under 5% CO_2.

To test TRAP-cage stability in the culture medium, purified sample was added to DMEM medium containing 0, 2 and 10% fetal bovine serum (FBS) and incubated at 37 °C under 5% CO_2 for 2 h, 6 h and 18 h. Samples were subsequently analyzed by native PAGE followed by Instant blue gel staining (**Fig. 7a**).

Cell viability after TRAP-cage treatment was determined using the alamarBlue test (VWR). Cells were cultured in 96-well plates at a density of 2.5×10^4 cells per well. Next, cells were treated with 5 µg (0.6 nM) TRAP-cage, TRAP-cage filled with GFP(-21) and decorated with Alexa-647 and PTD4 in 50 mM HEPES with 150 mM NaCl pH 7.5 supplemented with 10% FBS for 4 h. After the treatment, 10 µl of alamarBlue

diluted in 90 µl DMEM medium was added per well, and cells were incubated for the next 3 h at 37 °C under 5% CO_2. Resazurin, the active component of alamarBlue, was reduced to the highly fluorescent compound resorufin only in viable cells and absorbance (excitation 570 nm, emission 630 nm) of this dye was recorded. Nontreated cells were used as a negative control (**Fig. 7b**). All samples were measured in triplicates, in three independent experiments.

Example 4. Delivery of Protein Cargo to Cells.

Delivery of TRAP-cage to cells was studied using human cancer cell lines MCF-7 and HeLa. Cells were incubated for different time periods with the purified TRAP-cages containing encapsulated GFP(-21) and labelled with Alexa-647 only or with Alexa-647 and PTD4 and analysed by flow cytometry. The fluorescent signal due to both Alexa-647 and GFP increased with prolonged incubation time in both cell lines treated with TRAP-cage with GFP labelled with Alexa-647 and PTD4 peptide (**Figure 3a, b, c**). These results show that external modification of TRAP-cages with cell penetrating peptides promote their cell entry and effective cargo delivery. Interestingly, this effect was more pronounced in the case of the MCF-7 cell line compared to the HeLa cell line (**Figure 8a, b, c**).

In order to discriminate between fluorescent signals from TRAP-cages which were internalized in the cells and those which were adsorbed externally on the cell membrane, confocal microscopy was used. TRAP-cage containing GFP(-21) and labelled with Alexa-647 but lacking PTD4 were not observed in the cells. In contrast, TRAP-cage containing GFP(-21) and decorated with PTD4 showed a clear signal in the cell interior 4 h after stimulation (**Figure 3d** and **Figure 8d**).

Example 5. Intracellular dynamics of TRAP-cage.

The high stability of TRAP-cage coupled with its ability to break apart in presence of modest concentrations of cellular reducing agents suggests that TRAP-cage in the cytoplasm should readily disassemble, releasing GFP(-21) cargo. As TRAP-cage and GFP possess discrete and trackable signals we hypothesized that cage disassembly and release of GFP(-21) may be strongly inferred if the Alexa-647 and GFP signals became non-colocalised after cell entry. To assess this possibility, we tracked both signals over time after addition to MCF-7 and HeLa cancer cells. Notably, in both cell lines tested, during the first 90 minutes of incubation, TRAP-cage was mainly present at the cell boundaries as indicated by the strong localisation of the Alexa-647 signal there (**Figure 4a, 9a**) and the GFP signal was barely detectable (**Figure 4b, 9b**). However, after 3 h of incubation, the TRAP-cage signal (Alexa-647) became weaker

and appeared to be distributed more evenly in the cell, whereas the GFP signal was clearly detectable, due likely to its release from the TRAP-cages (**Figure 4a, b** and **Figure 9a, b**).

Example 6. Influence of Alexa-647 of GFP(-21) fluorescence

To assess the potential influence of Alexa-647 on GFP(-21) fluorescence (suggested by **Fig. 6a**, middle panel) we compared, by confocal microscope imaging, TRAP-cages filled with cargo where the cages compared were either decorated with PTD4 peptide only, or were fully decorated (PTD4 and Alexa-647) (**Fig. 10a**). Briefly, cells were treated with the respective samples as described in Materials and Methods. Next, cells were fixed and stained following the protocol described above. The fluorescence intensity in the green channel was quantified with ImageJ. Calculations of the mean fluorescence intensity (**Fig. 10b**) took into account the background signal from each field of view.

Additionally, in-solution fluorescence of GFP(-21) encapsulated in the fully decorated TRAP-cage was compared to the fluorescence of the cargo in the TRAP-cage without Alexa-647 using a RF-6000 Shimadzu® Spectro Fluorophotometer. As shown in **Fig. 10c** presence of the Alexa-647 dye on the TRAP-cage results in approximately 30% reduction in the fluorescence of its cargo.

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CLAIMS

1. An artificial TRAP-cage comprising a selected number of TRAP rings and encapsulated therein a guest cargo.

2. The cage according to claim 1 wherein the guest cargo is selected from the group comprising a nucleic acid, an enzyme, a therapeutic agent, a small molecule, organic or inorganic nanoparticles, a peptide, a metal, an antigen, an antibody and toxin and fragments thereof of all the foregoing that are of therapeutic value.

3. The cage according to claim 2 wherein the nucleic acid is selected from the group comprising DNA, RNA, mRNA, siRNA, tRNA and micro-RNA.

4. The therapeutic agent according to 2 wherein the enzyme is an enzyme associated with an over-expression in a metabolic disorder or disease or an under-expression in a metabolic disorder or disease.

5. The enzyme according to claim 4 wherein the enzyme is selected from the group comprising hydrogenase, dehydrogenase, lipase, lyase, ligase, protease, transferase, reductase, recombinase and nuclease acid modification enzyme.

6. The therapeutic agent according to claim 2 wherein the therapeutic agent is selected from the group comprising a cancer therapeutic, an anti-infection therapeutic, a vascular disease therapeutic, an immune therapeutic, senolytic and a neurological therapeutic.

7. The metal according to claim 2 wherein the metal is selected from the group comprising iron, zinc, platinum, copper, sodium, cadmium, lanthanide, gadolinium, technetium, calcium, potassium, chromium, magnesium, molybdenum and salts or complexes thereof.

8. The toxin according to claim 2 wherein the toxin is selected from the group comprising a ligand targeted toxin, a protease activated toxin, melittin and a toxin-based suicide gene therapeutic.

9. The cage according to any preceding claim wherein the internal guest cargoes are the same or different from one another.

10. The cage according to any preceding claim further including at least one external decoration.

11. The cage according to claim 10 wherein at least one of the external decorations comprises a cell penetrating agent to promote intracellular delivery of the cage containing an internal guest cargo.

12. The cage according to claim 11 wherein the cell penetrating agent is PTD4.

13. The cage according to any preceding claim wherein the number of TRAP rings in the TRAP-cage is between 6 to 60.

14. The TRAP-cage according to claim 13 wherein the number of TRAP rings in the TRAP-cage is 24.

15. The TRAP-cage according to any claim wherein opening of the cage is programmable.

16. The TRAP-cage according to claim 15 wherein the programmable opening of the cage is dependent on selection of a molecular or atomic cross-linker which hold the TRAP-rings in place in the TRAP-cage.

17. The TRAP-cage according to claim 16 wherein the molecular cross-linker is either (i) a reduction responsive/sensitive linker, whereby the cage opens under reduction conditions; or (ii) a photo-activatable linker whereby the cage opens upon exposure to light.

18. Use of the artificial TRAP-cage according to any preceding claim as a delivery vehicle for intracellular delivery of its internal guest cargo.

19. Use of the artificial TRAP-cage according to any one of claims 1 to 17 as a vaccine.

20. Use of the artificial TRAP-cage according to any one of claims 1 to 17 for the treatment of an illness or disease condition selected from the group comprising cancer, vascular disease, cardiovascular disease, diabetes, infection, auto-immune condition, neurodegenerative disease, cellular senescence disease, arthritis and respiratory disease.

21. A method of making an artificial TRAP-cage with an encapsulated guest cargo, the method comprising:

(i) obtaining TRAP ring units by expression of the TRAP ring units in a suitable expression system and purification of the said units from the expression system;

(ii) conjugation of the TRAP ring units via at least one free thiol linkage with a molecular cross-linker;

(iii) modification of the TRAP ring units to provide a suitable interior surface environment for capturing a guest cargo;

(iv) formation of the TRAP-cage by self-assembly to provide a cage lumen wherein the guest cargo is encapsulated; and

(v) purification and isolation of the TRAP-cages encapsulating the guest cargo.

22. The method of claim 21 wherein the modification of step (iii) is selected from the group comprising:

(i) super charging the interior surface of the TRAP-cage lumen;

(ii) genetic fusion of the guest cargo to an interior surface of the TRAP-cage lumen;

(iii) SpyCatcher/SpyTag conjugation of the guest cargo to an interior surface of the TRAP-cage lumen; and

(iv) via covalent bond formation in both chemical and enzymatic methods.

23. The method according to claim 22 wherein the super charging of step (i) of the interior surface provides either a net positive or net negative charge on the interior surface of the cage lumen.

24. The method according to claim 22 wherein the TRAP rings are variants.

25. The method according to claim 24 wherein the variant is either TRAP $^{K35C\ E48Q}$ or TRAP $^{K35C\ E48K}$

26. The method according to any of claim 25 wherein the cage formation step of part (iii) for TRAP $^{K35C\ E48Q}$ is performed in sodium bicarbonate buffer at pH 9-11.

27. The method according to any of claim 25 wherein the cage formation step of part (iii) for TRAP $^{K35C\ E48k}$ is performed in sodium bicarbonate buffer at pH 10-10.5.

28. The method according to any one of claims where the guest cargo can be loaded either pre or post assembly of the TRAP-cage.

29. The method according to claim 22 wherein the genetic fusion of the guest cargo to an interior surface of the TRAP-cage lumen of step (ii) is via N-terminus fusion of the guest cargo to an N-terminus of TRAP ^{K35C} which faces into the interior surface of the lumen.

30. The method according to claim 22 wherein the SpyCatcher/ SpyTag conjugation of the guest cargo to an interior surface of the TRAP-cage lumen of step

(iii) wherein the SpyCatcher is introduced in a loop region of TRAP rings between residues 47 and 48, which faces to the interior when assembled into TRAP-cages and the guest cargo contains a SpyTag.

31. The method of claim 22 wherein enzymatic modification is via peptide ligase selected from the group comprising sortases, asparaginyl endoproteases, trypsin related enzymes and subtilisin-derived variants and covalent chemical bond formation may include strain promoted alkyne-azide cycloaddition and pseudopeptide bonds.

32. A method of treatment of an individual in need of therapy suffering from a condition selected from the group comprising cancer, vascular disease, cardiovascular disease, diabetes, infection, auto-immune condition, neurodegenerative and neurological disease, cellular senescence disease, arthritis and respiratory disease, the method comprising administering a therapeutically effective amount of an artificial TRAP-cage bearing one or more internal guest cargo selected from the group comprising a nucleic acid, an enzyme, a therapeutic agent, a small molecule, organic or inorganic nanoparticles, a peptide, a metal, an antigen, an antibody and toxin and fragments thereof of all the foregoing that are of therapeutic value.

33. A method of vaccinating an individual in need of vaccination from a condition selected from the group comprising cancer, vascular disease, cardiovascular disease, diabetes, infection, auto-immune condition, neurodegenerative and neurological disease, cellular senescence disease, arthritis and respiratory disease, the method comprising administering a therapeutically effective amount of an artificial TRAP-cage bearing one or more internal guest cargo selected from the group comprising a nucleic acid, an enzyme, a therapeutic agent, a small molecule, organic or inorganic nanoparticles, a peptide, a metal, an antigen, an antibody and toxin and fragments thereof of all the foregoing that are of therapeutic value

34. The methods of either claims 32 or 33 wherein the TRAP-cage therapeutic is administered via intranasal inhalation or injection.

1. Cage à piège TRAP artificielle comprenant un nombre sélectionné d'anneaux TRAP et encapsulant à l'intérieur une cargaison invitée.

2. La cage selon la revendication 1 dans laquelle la cargaison invitée est choisie dans le groupe comprenant un acide nucléique, une enzyme, un agent thérapeutique, une petite molécule, des nanoparticules organiques ou inorganiques, un peptide, un métal, un antigène, un anticorps et toxine et des fragments de tout ce qui précède qui ont une valeur thérapeutique.

3. La cage selon la revendication 2 dans laquelle l'acide nucléique est choisi dans le groupe comprenant l'ADN, l'ARN, l'ARNm, l'ARNsi, l'ARNt et le micro-ARN.

4. L'agent thérapeutique selon la revendication 2 dans lequel l'enzyme est une enzyme associée à une surexpression dans un trouble ou une maladie métabolique ou une sous-expression dans un trouble ou une maladie métabolique.

5. L'enzyme selon la revendication 4, dans laquelle l'enzyme est choisie dans le groupe comprenant l'hydrogénase, la déshydrogénase, la lipase, la lyase, la ligase, la protéase, la transférase, la réductase, la recombinase et l'enzyme de modification d'acide nucléase.

6. L'agent thérapeutique selon la revendication 2, dans lequel l'agent thérapeutique est choisi dans le groupe comprenant un agent thérapeutique anticancéreux, un agent thérapeutique anti-infectieux, un agent thérapeutique contre les maladies vasculaires, un agent thérapeutique immunitaire, un agent thérapeutique sénolytique et un thérapeutique neurologique.

7. Le métal selon la revendication 2 dans lequel le métal est choisi dans le groupe comprenant le fer, le zinc, le platine, le cuivre, le sodium, le cadmium, le lanthanide, le gadolinium, le technétium, le calcium, le potassium, le chrome, le magnésium, le molybdène et leurs sels ou complexes.

8. La toxine selon la revendication 2, dans laquelle la toxine est choisie dans le groupe comprenant une toxine ciblée par un ligand, une toxine activée par une protéase, de la mélittine et un gène thérapeutique à base de toxine.

9. La cage selon l'une quelconque des revendications précédentes, dans laquelle les cargaisons internes invitées sont identiques ou différentes les unes des autres.

10. La cage selon l'une quelconque des revendications précédentes comprenant en outre au moins une décoration extérieure.

11. La cage selon la revendication 10, dans laquelle au moins une des décorations externes comprend un agent de pénétration cellulaire pour favoriser la délivrance intracellulaire de la cage contenant une cargaison interne invitée.

12. La cage selon la revendication 11, dans laquelle l'agent de pénétration cellulaire est le PTD4.

13. La cage selon l'une quelconque des revendications précédentes, dans laquelle le nombre d'anneaux TRAP dans la cage TRAP est compris entre 6 et 60.

14. La cage à piège TRAP selon la revendication 13, dans laquelle le nombre d'anneaux TRAP dans la cage TRAP est de 24.

15. La cage à piège TRAP selon l'une quelconque des revendications, dans laquelle l'ouverture de la cage est programmable.

16. La cage à piège TRAP selon la revendication 15, dans laquelle l'ouverture programmable de la cage dépend du choix d'un agent de réticulation moléculaire ou atomique qui maintient les anneaux TRAP en place dans la cage TRAP.

17. La cage à piège TRAP selon la revendication 16, dans laquelle l'agent de réticulation moléculaire est soit (i) un agent de liaison répondant/sensible à la réduction, moyennant quoi la cage s'ouvre dans des conditions de réduction ; ou (ii) un agent de liaison photo-activable par lequel la cage s'ouvre lors d'une exposition à la lumière.

18. Utilisation de la cage à piège TRAP artificielle selon l'une quelconque des revendications précédentes comme véhicule de livraison pour la livraison intracellulaire de sa cargaison interne invitée.

19. Utilisation de la cage à piège TRAP artificielle selon l'une quelconque des revendications 1 à 17 comme vaccin.

20. Utilisation de la cage à piège TRAP artificielle selon l'une quelconque des revendications 1 à 17 pour le traitement d'une maladie ou d'un état pathologique choisi dans le groupe comprenant le cancer, les maladies vasculaires, les maladies cardiovasculaires, le diabète, les infections, les maladies auto-immunes, neurodégénératives maladie, maladie de sénescence cellulaire, arthrite et maladie respiratoire.

21. Procédé de fabrication d'une cage à piège TRAP artificielle avec une cargaison invitée encapsulée, le procédé comprenant :
    (i) obtenir des unités d'anneaux TRAP par expression des unités d'anneaux TRAP dans un système d'expression approprié et purification desdites unités à partir du système d'expression;
    (ii) conjugaison des unités d'anneaux TRAP via au moins une liaison thiol libre avec un agent de réticulation moléculaire;
    (iii) modification des unités d'anneaux TRAP pour fournir un environnement de surface intérieur approprié pour capturer une cargaison invitée;
    (iv) formation de la cage TRAP par autoassemblage pour fournir une cavité tubulaire dans la cage, dans laquelle la cargaison d'invité est encapsulée; et
    (v) purification et l'isolement des cages TRAP encapsulant la cargaison invitée.

22. Procédé selon la revendication 21, dans lequel la modification de l'étape (iii) est choisie dans le groupe comprenant :
    (i) super-charger la surface intérieure de la cavité tubulaire de la cage TRAP;
    (ii) fusion génétique de la cargaison invitée à une surface intérieure de la cavité tubulaire de la cage TRAP;
    (iii) conjugaison SpyCatcher/SpyTag de la cargaison invitée à une surface intérieure de la cavité tubulaire de la cage TRAP; et
    (iv) formation de liaisons covalentes dans des méthodes chimiques et enzymatiques.

23. Procédé selon la revendication 22, dans lequel la super-charge de l'étape (i) de la surface intérieure fournit une charge nette positive ou nette négative sur la surface intérieure de la cavité tubulaire de la cage.

24. Procédé selon la revendication 22 dans lequel les anneaux TRAP sont des variantes.

25. Procédé selon la revendication 24, dans lequel le variant est soit TRAP K35C E48Q, soit TRAP K35C E48K.

26. Procédé selon l'une quelconque des revendications 25, dans lequel l'étape de formation de cage de la partie (iii) pour TRAP K35C E48Q est réalisée dans un tampon de bicarbonate de sodium à un pH de 9 à 11.

27. Procédé selon l'une quelconque des revendications 25, dans lequel l'étape de formation de cage de la partie (iii) pour TRAP K35C E48k est réalisée dans un tampon de bicarbonate de sodium à un pH de 10 à 10,5.

28. Procédé selon l'une quelconque des revendications, dans lequel la cargaison invitée peut être chargée soit avant soit après l'assemblage de la cage TRAP.

29. Procédé selon la revendication 22, dans lequel la fusion génétique de la cargaison invitée à une surface intérieure de la cavité tubulaire de la cage TRAP de l'étape (ii) se fait par fusion N-terminale de la cargaison d'invité à une extrémité N-terminale de TRAP K35C qui fait face à la surface intérieure de la cavité tubulaire.

30. Procédé selon la revendication 22, dans lequel la conjugaison SpyCatcher/SpyTag de la cargaison invitée à une surface intérieure de la cavité tubulaire de la cage TRAP de l'étape (iii) dans laquelle le SpyCatcher est introduit dans une région en boucle des anneaux TRAP entre les résidus 47 et 48, qui fait face à l'intérieur lorsqu'il est assemblé dans des cages TRAP et que la cargaison invitée contient un SpyTag.

31. Procédé selon la revendication 22, dans lequel la modification enzymatique se fait via peptide ligase choisi dans le groupe comprenant les sortases, les asparaginyl endoprotéases, les enzymes apparentées à la trypsine et les variants dérivés de subtilisine et la formation de liaisons chimiques covalentes peut inclure une cycloaddition alcyne-azide favorisée par la souche et des liaisons pseudopeptidiques.

32. Méthode de traitement d'un individu nécessitant une thérapie et souffrant d'un état choisi dans le groupe comprenant le cancer, les maladies vasculaires, les maladies cardiovasculaires, le diabète, les infections, les maladies auto-immunes, les maladies neurodégénératives et neurologiques, la sénescence cellulaire, l'arthrite et une maladie respiratoire, le procédé comprenant l'administration d'une quantité thérapeutiquement efficace d'une cage à piège TRAP artificielle portant une ou plusieurs cargaisons invitées internes sélectionnées dans le groupe comprenant un acide nucléique, une enzyme, un agent thérapeutique, une petite molécule, des nanoparticules organiques ou inorganiques,

un peptide, un métal, un antigène, un anticorps et une toxine et leurs fragments de tout ceLU102571 qui précède qui ont une valeur thérapeutique.

33. Procédé de vaccination d'un individu nécessitant une vaccination à partir d'un état choisi dans le groupe comprenant le cancer, les maladies vasculaires, les maladies cardiovasculaires, le diabète, les infections, les maladies auto-immunes, les maladies neurodégénératives et neurologiques, les maladies de sénescence cellulaire, l'arthrite et les maladies respiratoires. , le procédé consistant à administrer une quantité thérapeutiquement efficace d'une cage à piège TRAP artificielle portant une ou plusieurs cargaisons invitées internes choisies dans le groupe comprenant un acide nucléique, une enzyme, un agent thérapeutique, une petite molécule, des nanoparticules organiques ou inorganiques, un peptide, un métal, un antigène, un anticorps et une toxine et leurs fragments de tout ce qui précède qui ont une valeur thérapeutique.

34. Procédé selon l'une ou l'autre des revendications 32 ou 33, dans lequel l'agent thérapeutique de la cage TRAP est administré par inhalation ou injection intranasale.

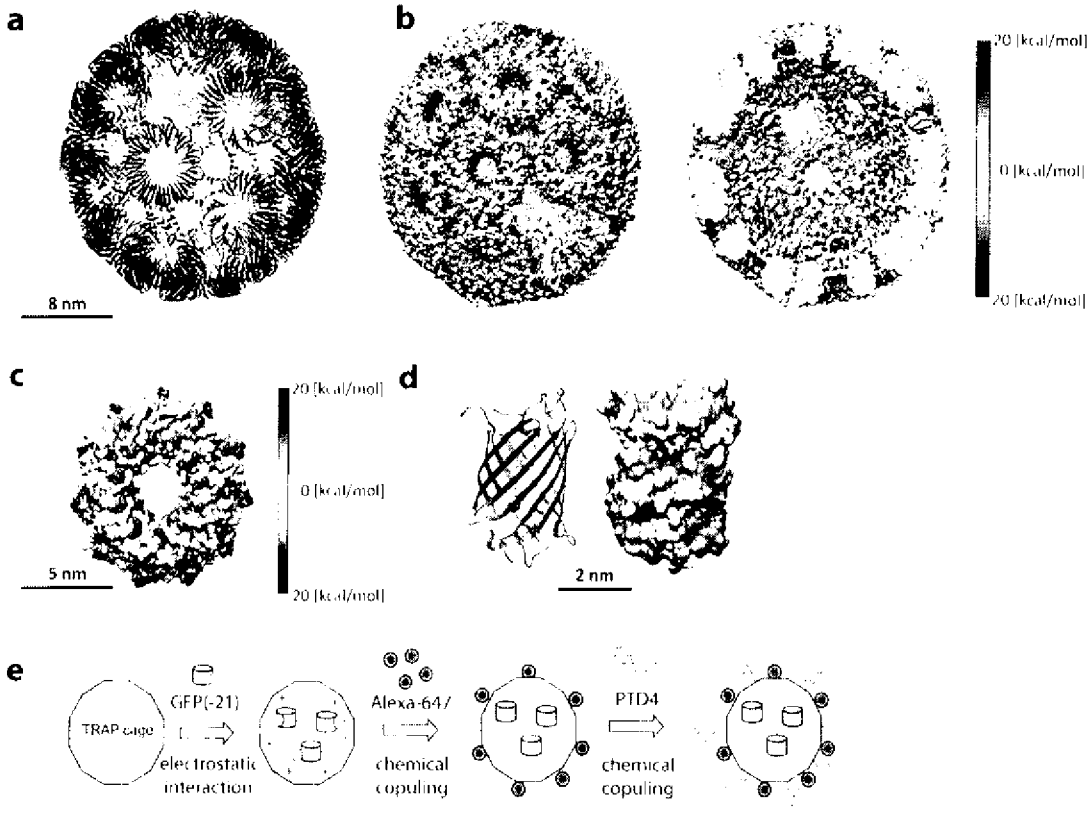
a
8 nm
b
20 [kcal/mol]
0 [kcal/mol]
-20 [kcal/mol]
c
20 [kcal/mol]
0 [kcal/mol]
-20 [kcal/mol]
5 nm
d
2 nm
e
TRAP cage
GFP(-21)
electrostatic interaction
Alexa-647
chemical copuling
PTD4
chemical copuling

Fig. 1

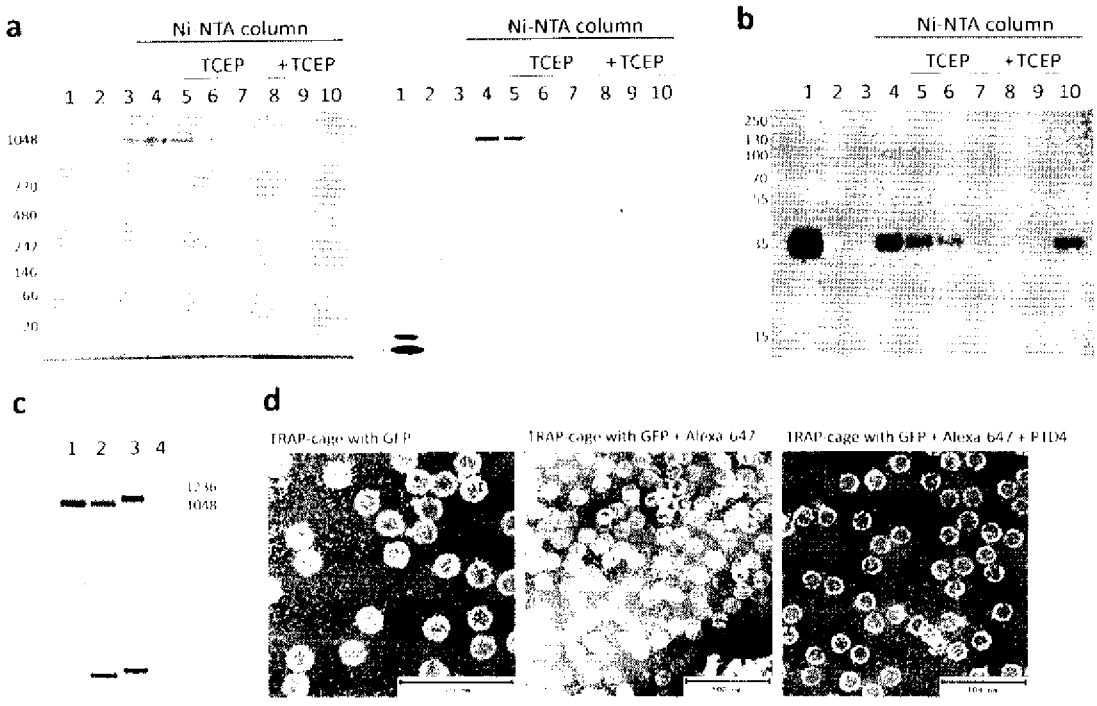
a
Ni-NTA column
TCEP
+TCEP
1 2 3 4 5 6 7 8 9 10
1048
480
146
66
Ni-NTA column
TCEP
+TCEP
1 2 3 4 5 6 7 8 9 10
b
Ni-NTA column
TCEP
+TCEP
1 2 3 4 5 6 7 8 9 10
250
130
100
70
55
35
15
c
1 2 3 4
1236
1048
d
TRAP-cage with GFP
TRAP-cage with GFP + Alexa 647
TRAP-cage with GFP + Alexa 647 + PTD4

Fig. 2

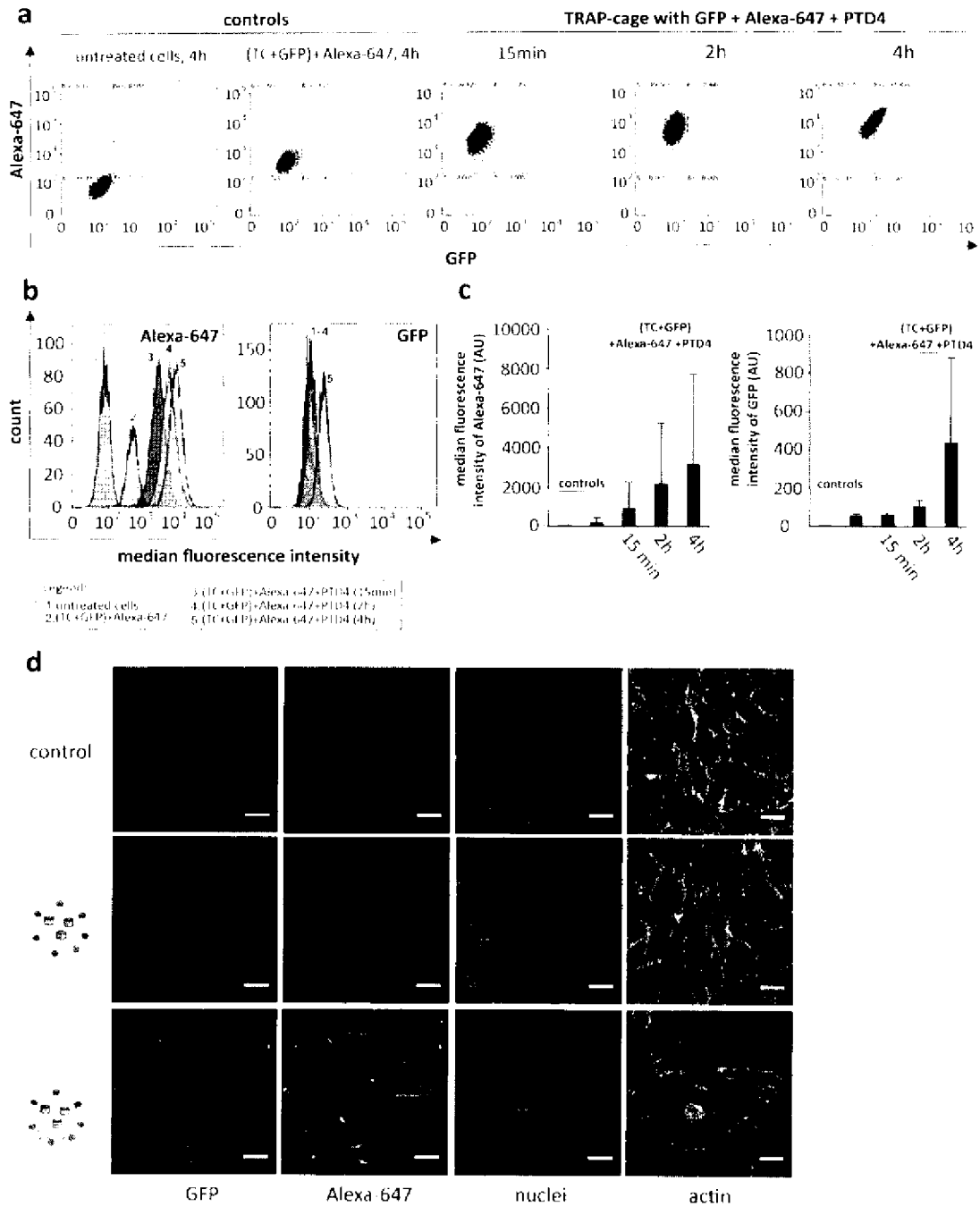
a
controls
TRAP-cage with GFP + Alexa-647 + PTD4
untreated cells, 4h
(TC+GFP)+Alexa-647, 4h
15min
2h
4h
Alexa-647
GFP
b
Alexa-647
GFP
count
median fluorescence intensity
c
median fluorescence intensity of Alexa-647 (AU)
median fluorescence intensity of GFP (AU)
(TC+GFP) +Alexa-647 +PTD4
controls
15 min
2h
4h
d
control
GFP
Alexa 647
nuclei
actin

Fig. 3

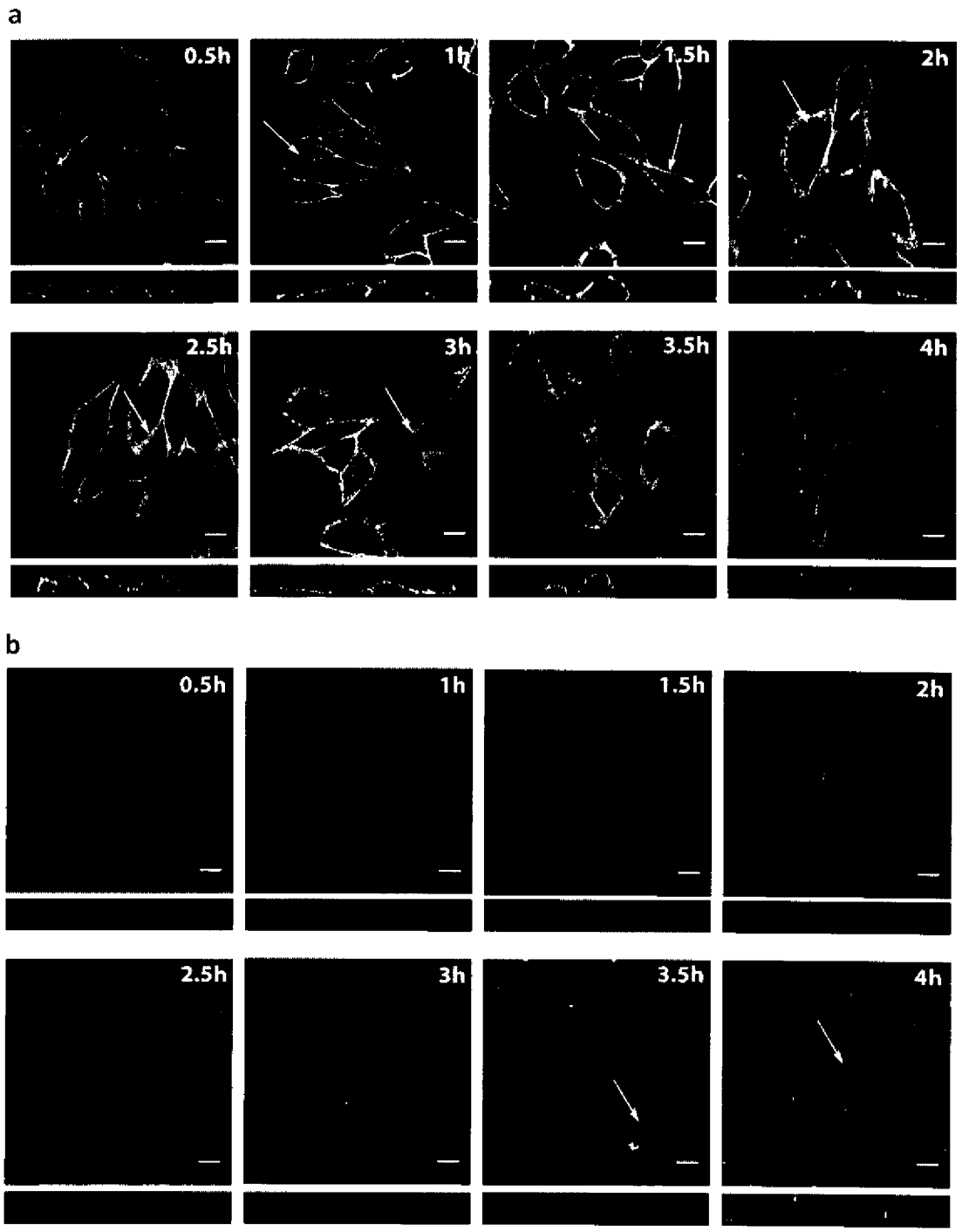

Fig. 4

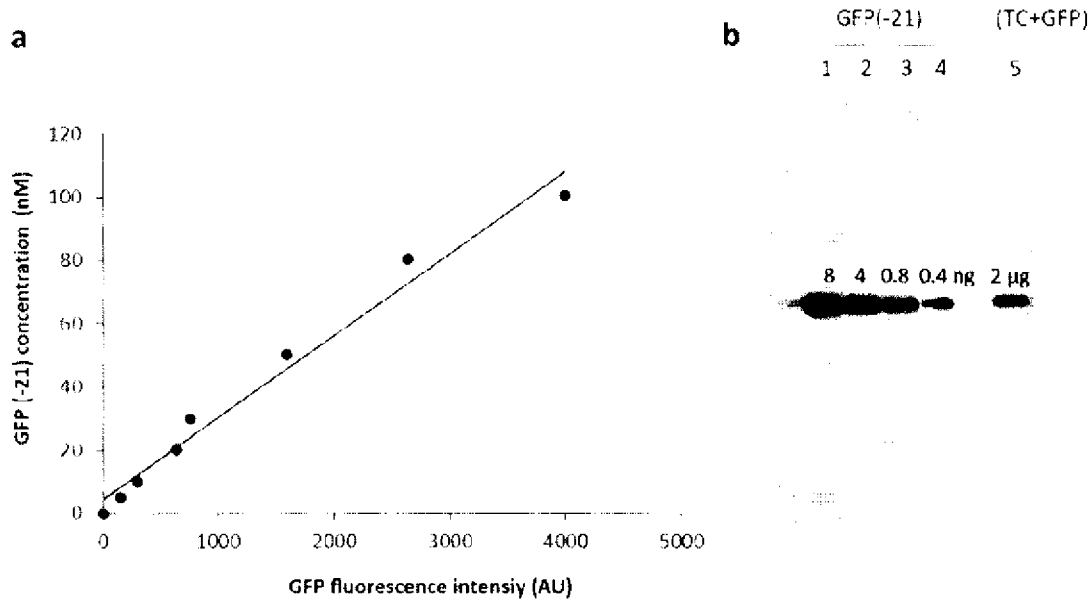
a
b
GFP(-21)
(TC+GFP)
1 2 3 4 5
8 4 0.8 0.4 ng 2 µg
GFP (-21) concentration (nM)
GFP fluorescence intensiy (AU)
0
20
40
60
80
100
120
1000
2000
3000
4000
5000

Fig. 5

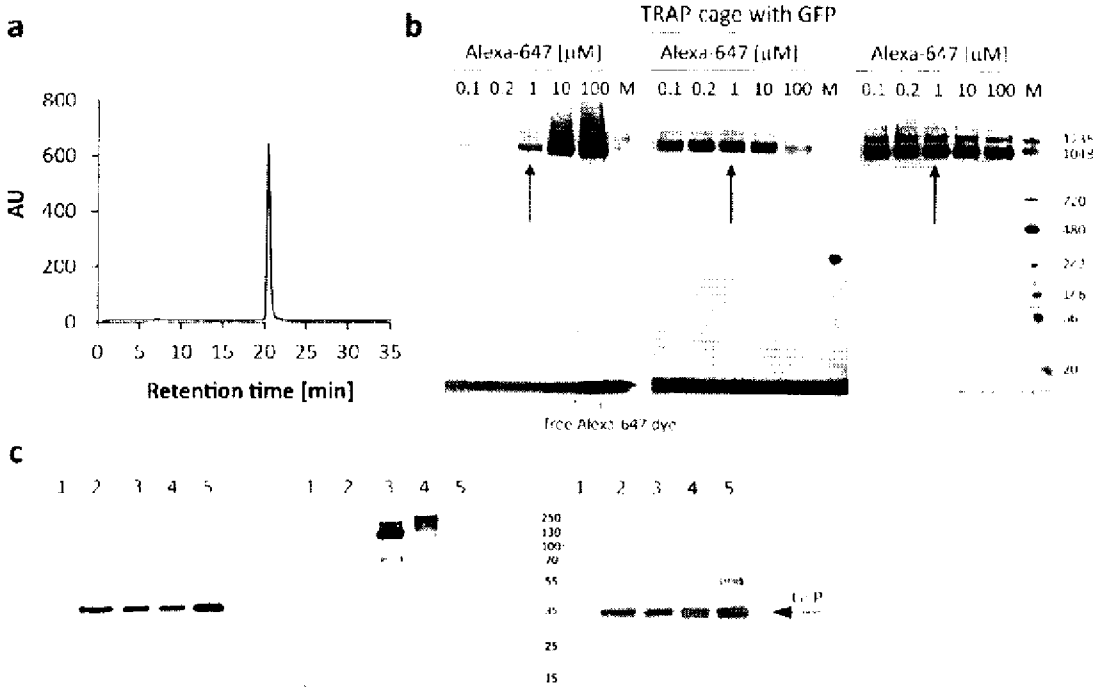
a
b
c
TRAP cage with GFP
Alexa-647 [µM]
0.1 0.2 1 10 100 M
AU
Retention time [min]
free Alexa 647 dye
1 2 3 4 5
250
130
100
70
55
40
35
25
15

Fig. 6

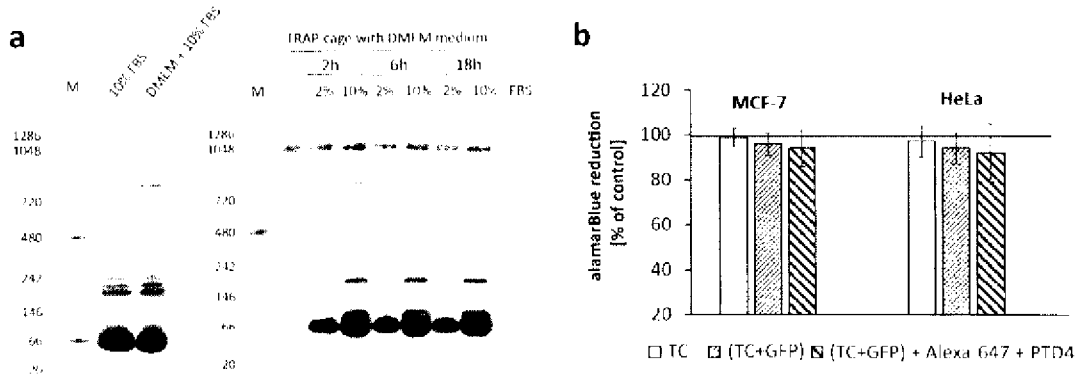
a
M
10% FBS
DMEM + 10% FBS
TRAP cage with DMEM medium
2h
6h
18h
2% 10% 2% 10% 2% 10% FBS
1286
1048
720
480
242
146
66
20
b
MCF-7
HeLa
alamarBlue reduction
[% of control]
120
100
80
60
40
20
TC
(TC+GFP)
(TC+GFP) + Alexa 647 + PTD4

Fig. 7

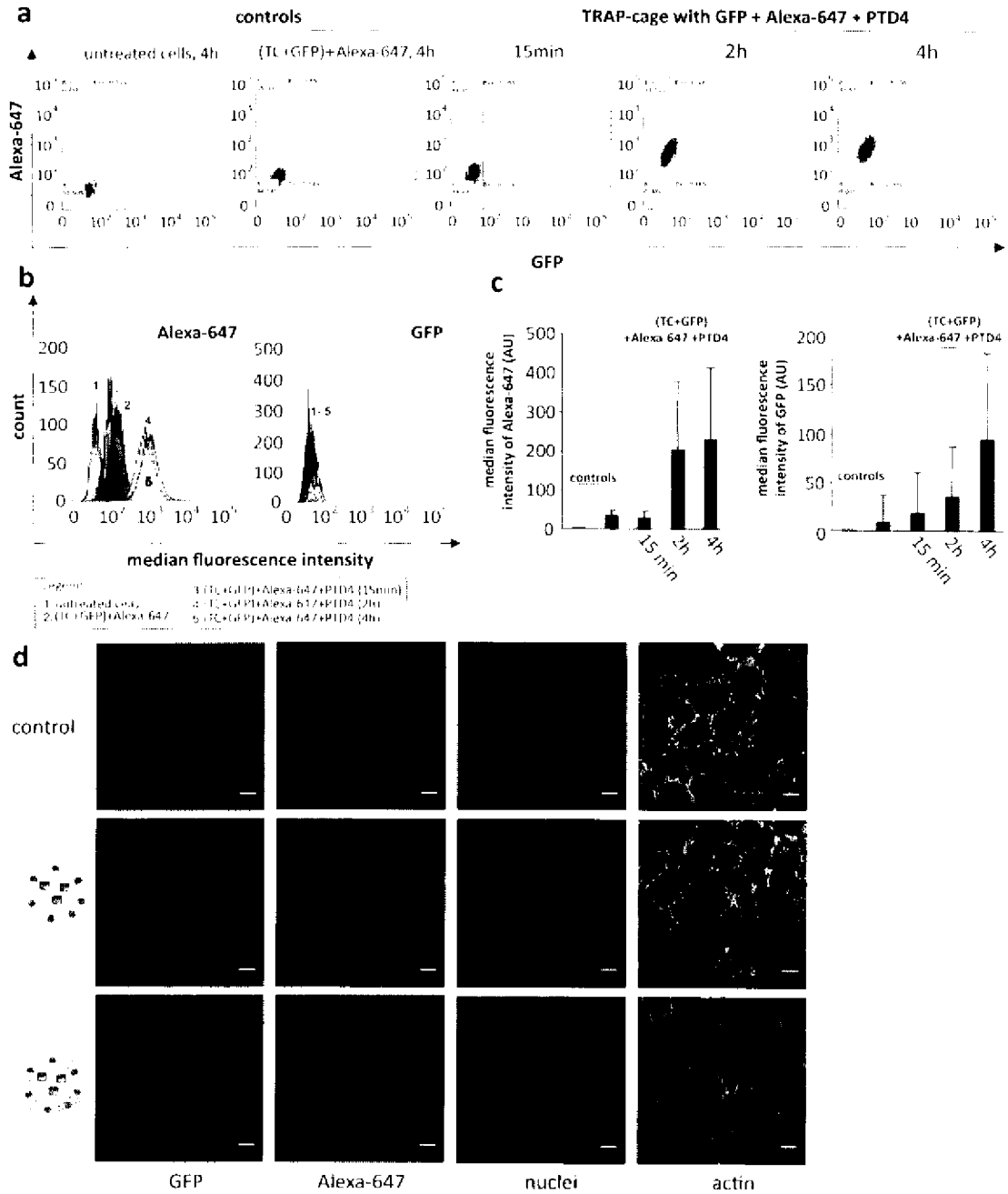
a
controls
TRAP-cage with GFP + Alexa-647 + PTD4
untreated cells, 4h
(TC+GFP)+Alexa-647, 4h
15min
2h
4h
Alexa-647
GFP
b
Alexa-647
GFP
count
median fluorescence intensity
c
(TC+GFP) +Alexa 647 +PTD4
median fluorescence intensity of Alexa-647 (AU)
median fluorescence intensity of GFP (AU)
controls
15 min
2h
4h
d
control
GFP
Alexa-647
nuclei
actin

Fig. 8

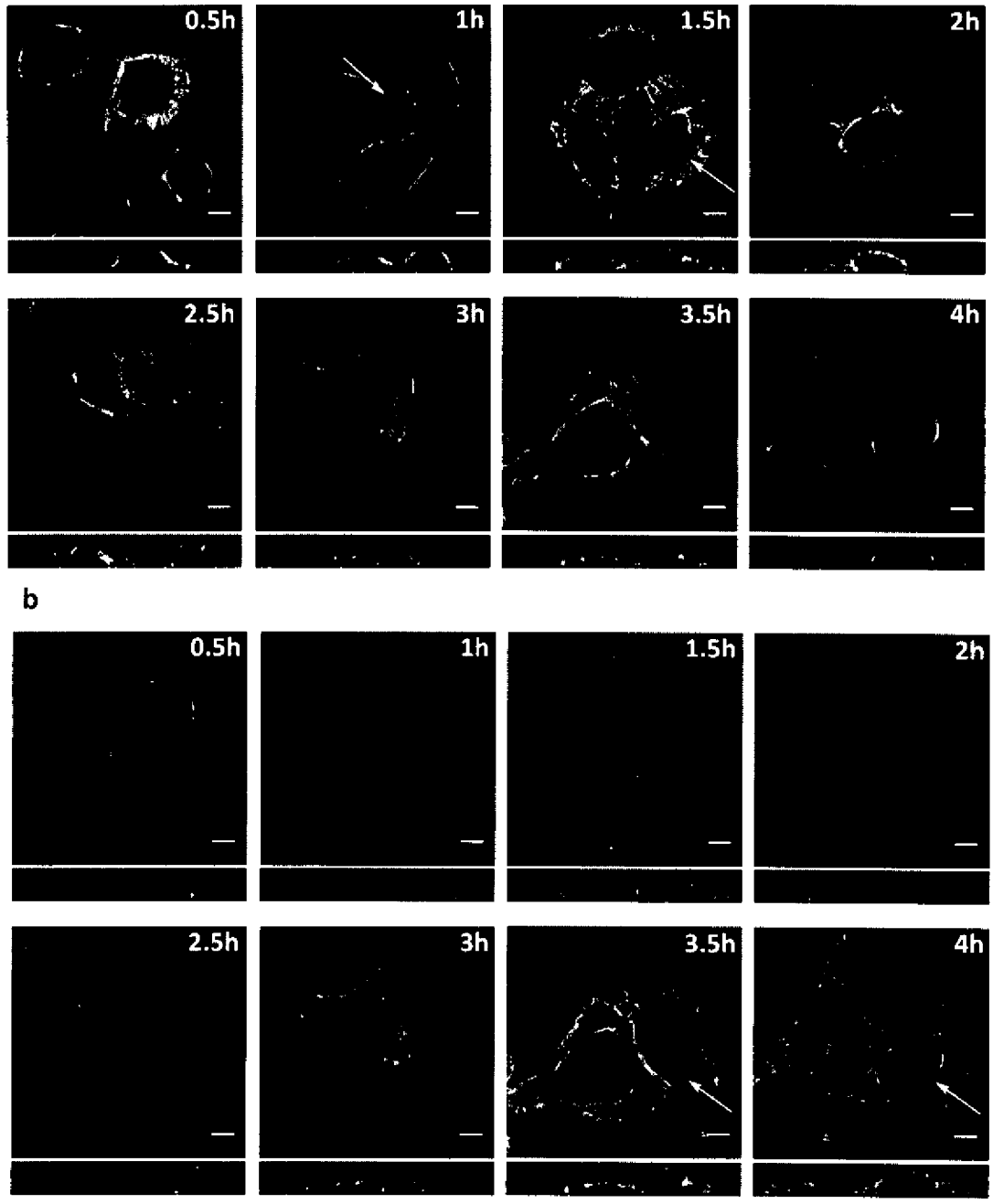

Fig. 9

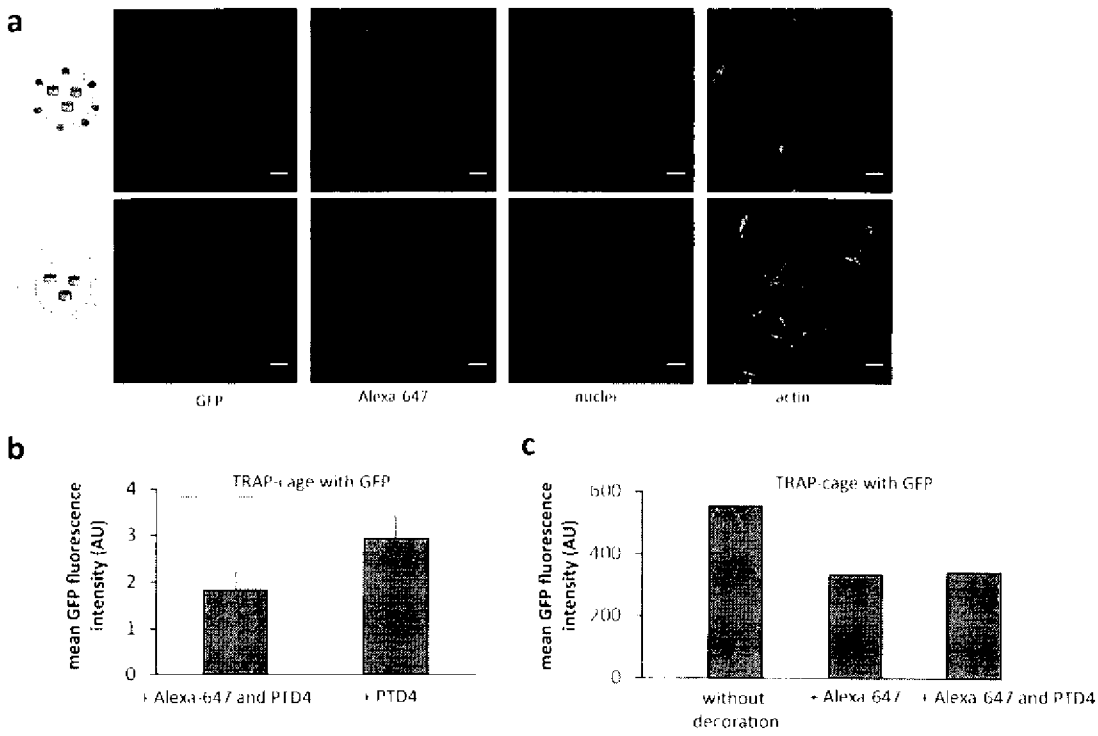
a
GFP
Alexa 647
nuclei
actin
b
TRAP-cage with GFP
mean GFP fluorescence intensity (AU)
4
3
2
1
0
+ Alexa-647 and PTD4
+ PTD4
c
TRAP-cage with GFP
mean GFP fluorescence intensity (AU)
600
400
200
0
without decoration
+ Alexa 647
+ Alexa 647 and PTD4

Fig. 10