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(54) Title: MODIFIED MscL PROTEIN CHANNEL

(57) Abstract: The invention relates to the field of drug delivery, in particular to compounds and methods for the chemical modification of a proteinaceous channel to be used in pharmaceutical delivery vehicles for controlled and/ or localised release of therapeutic molecules (e.g. small molecules, peptides, proteins or other macromolecules). Provided are pH- and/or light-responsive compounds capable of controlling the channel activity of a mechanosensitive channel, such as the MscL channel protein of E.coli, or a functional equivalent thereof, and use of these compounds to convert a mechanosensitive channel protein into a pH- and/or light-responsive channel. Also provided are drug delivery vehicles comprising a pH- and/or light-responsive channel protein.

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Title: Modified MscL protein channel

5 The invention relates to the field of drug delivery. The invention particularly relates to compounds and methods for the chemical modification of a proteinaceous substance, such as a channel protein, suitable for use in pharmaceutical delivery vehicles for controlled and/ or localised release of therapeutic molecules (e.g. small molecules, peptides, proteins or other macromolecules).

10 After their discovery in 1965 (Bangham, A. D., et al., J. Mol. Biol. 1965, 13, 238-253), liposomes became a promising tool for the drug delivery. Since that period, many methodologies have been developed for liposomal compositions, efficient drug encapsulation and retention, stability and targeting (Woodle, M. C., Lasic, D. D., Biochim. Biophys. Act. 1992, 1113, 171- 199). After being loaded with the desired
15 amount of drug and being stable enough to accumulate in the target site, the next step generally is to release the content of the liposomes in response to a specific stimulus at the target. It has been hypothesised that enhanced release at the target site will significantly improve the specificity and efficacy of a liposomal drug (Drummond, D. C., et al., Pharmacol. Rev. 1999, 51, 691-744; Bally., M. B., et al., J.
20 Liposomes Res. 1998, 8, 299- 335; Fenske, D. B., at al., Curr. Opin. Mol. Ther. 2001, 3, 153- 158).

 Various strategies have been used for triggering liposomal release at the target site. Formation of channels and defects in the liposomal bilayer, lamellar-micellar or lamellar-hexagonal phase transition, lipid phase separation and liposome
25 fusion are some examples (Meers, P. Adv. Drug Deliv. Rev. 2001, 53, 265- 272; Drummond, D. C., et al., Prog. Lipid Res. 2000, 39, 409- 460; Asokan, A., Cho, M. J., J. Pharm. Sci. 2002, 91, 903- 913; Chu, C. J, Szoka, F. C., J. Liposome Res. 1994, 4, 361- 395; Thomas, J. L., Tirrel, D. A., Acc. Chem. Res. 1992, 25, 336- 342). All these efforts have been focused on the lipid components of the liposomes, and a very limited
30 success has been achieved (Guo, X., Szoka F. C. Acc. Chem. Res. 2003, 36, 335- 341).

 The present invention provides a set of novel compounds which, upon attachment to a proteinaceous valve (protein channel), confer a specific sensitivity to the modified

protein channel such that the channel responds to specific stimuli present at the target site (e.g. pH, light), leading to opening of the channel. A modified protein channel of the invention is advantageously used in a drug delivery vehicle (such as liposomes) to achieve a controlled and localised unloading of the contents of the vehicle at a target site, such as a tumour.

Provided is a protein that is modified with a pH-responsive compound of the general formula Q-C, wherein Q is a chargeable group selected from the chargeable groups Q₁, Q₂, and Q₃, and wherein C is a coupling group selected from the coupling groups C₁, C₂, C₃, C₄, C₅ and C₆ as depicted in Figure 1. The coupling group C allows covalent attachment of the compound to a protein. Thus, a compound according to the invention may have the general formula Q-C, wherein Q is any group selected from Q₁-Q₃ and wherein C is any group selected from C₁-C₆. The chargeable group Q can be either in a charged or uncharged state, depending on the local pH environment. Also provided are proteins modified with a light-sensitive compound comprising a photocleavable chargeable group P and a coupling group C. These compounds have the general formula P-C, wherein P is any group selected from P₁-P₈ and wherein C is any group selected from C₁-C₆. Preferably, a light-sensitive compound has the formula P₆-C₁ (see for example Example 5). In case any one of the coupling groups C₄, C₅ and C₆ is used, it is however also possible to incorporate two Q- or P-groups, or a combination of a Q and a P-group, in one compound. Examples of such compounds are 2(P₆)-C₄, 2(Q₁)-C₆, 2(P₁)-2(L₁)-C₅ and 2(Q₁)-2(L₁)-C₅.

A P-group can be regarded as a chargeable moiety provided with a photocleavable moiety which blocks charging of the chargeable moiety. Thus, in the absence of illumination a compound of the invention which comprises a P group is not chargeable. Only upon illumination of a P-containing compound, the photocleavable moiety of group P dissociates to release a chargeable moiety (see for example Figure 3). Similar to the Q groups described above, the chargeable moiety will be protonated at a pH lower than the pK_a of the chargeable moiety. Thus, the invention herewith provides pH-responsive compounds which only become pH-responsive upon illumination. The dual sensitivity of these compounds allows to modify a protein (channel) in such a manner that its activation can be tightly controlled in time.

In addition to groups Q (or P) and C, a compound may comprise a linker group L selected from the group of linker groups consisting of L₁-L₄ shown in Figure 1. The linker group connects the chargeable group Q or the photocleavable chargeable group P to the coupling group C. The general formula of those compounds with a
5 linker group is either Q-L-C or P-L-C. Preferably, the linker group is of the formula – (CH₂)_n– wherein n= 1-10 (i.e. group L₁), more preferably (CH₂)₂–.

In a preferred embodiment, a modified protein is a modified proteinaceous valve, herein also referred to as a modified channel protein. A protein may also be modified with more than one compound, for example with two or three identical
10 compounds or with different compounds. Essentially any type of proteinaceous substance may be modified as long as it contains at least one residue to which a coupling group C can be attached. The invention will be illustrated by the modification of a protein channel with a compound of the invention. However, other types of proteins can of course also be modified with a light- and/or pH-responsive
15 compound to confer a specific sensitivity to the protein that allows for controlling the functionality of the protein with local or external stimuli (pH, light). It has been shown for some other biological systems that a charged or polar substitution in the hydrophobic pore region of gated channels, like MscL, creates easily or constitutively gating channels. For instance, the Shaker Kv channel is a channel protein gating in
20 response to voltage changes in the membrane. Introduction of hydrophilic residues into the activation gate of Shaker Kv channels causes channel gating independent of the membrane voltage (Sukhareva, M., et al., 2003, J. Gen. Physiol. 122:541–556).

In a preferred embodiment, the coupling group C comprises an SSO₂CH₃ moiety (group C₃ in Fig. 1) to yield a methylthiosulfonate (MTS) conjugate. MTS
25 conjugates react very rapidly and specifically with cysteine sulfhydryls of a protein and are therefore particularly suitable as coupling group (see Figure 2). It has been previously shown that the G22C mutant of the MscL channel protein can be activated in spheroplasts (Yoshimura, K., et al., Biophys. J. 2001, 80, 2198- 2206) and growth cultures (Batiza et al., Proc. Natl. Acad. Sci. USA 2002, 99, 5643-5648) by the addition
30 of MTS reagents. The present invention now provides pH- and also light responsive methylthiosulfonate compounds (i.e. those comprising coupling group C₃), as well as pH- and light-responsive compounds with alternative coupling groups. Some pH-responsive compounds of the invention are designed to be protonated only at a pH

lower than approximately 7.4 (the physiological pH of mammals including humans), whereas others have a slightly higher pKa (typically around 7.9). Compound protonation introduces a charge into the constriction part of a mechanosensitive channel (e.g. the MscL channel protein), which in turn leads to channel opening. A
5 decreased pH can for example be found around solid tumors, liver cells, sites of inflammation and ischemic areas in the body.

As will be described below, of particular interest are pH-responsive compounds of the general formula $Q_1-L_1-C_3$. An example of such a compound is $R_1R_2N-CH_2-(CO)O-(CH_2)_2-S(SO_2)-CH_3$, wherein R_1 and R_2 are each independently H
10 or CH_3 , preferably wherein R_1 and R_2 are CH_3 . Figure 5 shows the structures of the compounds wherein R_1 and R_2 are both H (panel A; compound A: $Q_1[R_1=R_2=H]-L_1[n=2]-C_3$), wherein R_1 is H and R_2 is CH_3 (panel B; compound B: $Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$) and wherein R_1 and R_2 are both CH_3 (panel C; compound C: $Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$)

15 Examples of light-sensitive pH-responsive compounds of the invention can have the general formula P-C or P-L-C. It will be understood that, according to the invention, many different combinations can be made with the different photocleavable groups P_1-P_8 , coupling groups C_1-C_6 , and, optionally, linker groups L_1-L_4 . Preferred compounds of this type include compound $P_1[R_3=OMe, R_1=H]-L_1[n=1]-C_3$ (compound
20 D; Fig. 6) and compound $P_2[R_3=OMe, R_1=Me]-L_1[n=2]-C_3$ (compound F; Fig. 15). Another preferred light-sensitive compound is compound $P_6-C_1[X=I]$ (compound E), whose synthesis is described in Example 5 and schematically depicted in Figure 12. A light-sensitive or pH-responsive compound of the invention is easily attached to a protein, for example a mechanosensitive (MscL) channel protein, via the coupling
25 group C.

Also provided herein are methods for providing a compound according to the invention that involve standard step-by-step synthesis methods, reactions and reagents well known in the field of (medicinal) organic chemistry. The synthesis of a pH-responsive compound and a light-sensitive pH-responsive compound of the
30 invention is exemplified in Example 1 and 5 (see also Fig. 4, 6 and 12). The starting materials required for the synthesis are typically commercially available in high purity.

A further aspect of the invention relates to a method for providing a modified mechanosensitive channel of large conductance, preferably the MscL channel protein of *E. coli*, or a functional equivalent thereof. In nature the channel works as a membrane tension sensor. It protects cells from lysis under hypoosmotic shock conditions. Upon shock, water influx creates a turgor pressure in the cells and as a result, tension in the membrane increases. The MscL channel senses the increase in the membrane tension and forms an approximately 3.5 nm non-selective pore in the membrane and releases osmolytes to protect the cell from bursting (Sukharev S., I., *et al.*, Annu. Rev. Physiol. 1997, 59, 633- 657; Cruickshank, C., *et al.*, Biophys. J. 1997, 73, 1925- 1931). A method of the invention for providing a modified mechanosensitive channel protein, such as the MscL protein, or a functional equivalent thereof, comprises the chemical modification of the channel protein with a compound of the invention by the covalent attachment of said compound via the coupling group C to a target residue of said channel protein, preferably a cysteine residue, wherein said target residue is located in a region of said channel protein which upon introduction of a charge and/or a polar group induces opening of said channel. Functional equivalents of the MscL channel protein of *E. coli* comprise homologues of mechanosensitive channels from other organisms. The genes encoding MscL homologues from various prokaryotes are cloned (Moe, P.C., Blount, P. and Kung, C., Mol. Microbiol. 1998, 28, 583-592). Nucleic acid and amino acid sequences are available and have been used to obtain heterologous (over)-expression of several MscL proteins (Moe, P.C., *et al.* J. Biol. Chem., 2000, 40, 31121-31127). A useful MscL homologue can be found in *Lactococcus lactis*. Also, mutants or otherwise chemically altered MscL proteins can be used.

A light-sensitive or pH-responsive compound, or a compound that becomes pH-responsive after photocleavage provided herein is covalently attached to a target amino acid residue of a protein. The target residue may be naturally present or it may be artificially introduced into the protein using mutagenesis, preferably site-directed mutagenesis. Of course, the target residue of a protein to be modified by a compound of the invention is preferably located in or near a region that modulates the functionality of said protein. For example, in case of channel protein MscL, it is the region that controls the channel activity. The channel activity refers to the gating threshold, the frequency of change between the open, intermediate (if present) and

the closed states and the residence time in the open/ intermediate state (also known as dwell time) .

One region of interest for modifying a channel protein is the region that corresponds to the first cytoplasmic domain of MscL of *E.coli*. This region constitutes the N-terminal part of the protein (residues 1 - 14) and it is proposed that there might
5 be a supplementary gate formed by the first 9 amino acids of the N terminus, which occlude the channel even when the transmembrane helices have spread apart. (Sukharev, S., et al., Nature ,2001, 409, 720 - 724, Sukharev, S., et al, Biophysical J., 2001, 81, 917 - 936)). Another region corresponds to the first transmembrane domain,
10 TM1 helix, of *E.coli* MscL (residues 15 – 45). This is the most conserved region (Chang, G. et al., Science, 1998, 282, 2220 – 2226, Spencer, R. H., et al., Curr. Opin. Struct. Biol., 1999, 9, 448 – 454) within the MscL family.

Another region useful for modification may be the loop region (residues 46– 75). Mutagenesis studies have implicated this region as being important in MscL
15 gating (Blount, P., et al., Proc. Natl. Acad. Sci. USA ,1996, 93,11652-11657, Maurer, J. A., et al., J. Biol.Chem., 2000, 275, 22238 – 22244, Ajouz et al., J. Biol. Chem., 2000, 275: 1015-1022, Gu et al., Biophys. J., 1998, 74, 2889-2902). Observation of a significant number of loss-of-function mutants in this region recently led to the hypothesis that the loop serves as a spring connecting the first and the second
20 transmembrane domains (Maurer *et al.*, J. Biol. Chem., 2003, 278, 21076-21082). Besides gain-of-function and loss-of-function mutations, there are also mutations in the loop region that are known to alter the channel kinetics and sensitivity. For example, mutation Gln56His led to changes in channel kinetics and tension sensitivity depending on the pH on the periplasmic but not cytoplasmic side of the
25 membrane (Blount *et al.*, The EMBO J., 1996, 15, 4798-4805, Blount, P., *et al.*, Proc. Natl. Acad. Sci. USA ,1996, 93,11652-11657). When glutamine 56 is mutated to proline it causes changes in the gating sensitivity. Replacement of the 46th position (normally a glycine residue) with aspartic acid creates a severe gain-of-function mutant (Ou, X., *et al.*, Proc. Natl. Acad. Sci. USA, 1998, 95, 11471 – 11475). Yet
30 another region of interest for modification corresponds to amino acid residues 76-100 of the MscL protein.

Of particular interest is the modification of a target residue in the region that corresponds to around position 23 of the MscL protein of *E. coli*. Mutagenesis

experiments have shown the region located around Val 23 to be structurally and functionally very important (Perozo, E., *et al.*, Nature, 2002, 418, 942 – 948, Ou, X., *et al.*, Proc. Natl. Acad. Sci. USA, 1998, 95, 11471 – 11475, Maurer, J. A., *et al.*, J. Biol.Chem., 2000, 275, 22238 – 22244). In addition, it has been shown that mutation
5 of residue K31 can lead to changes in MscL gating (Blount, P., *et al.*, Proc. Natl. Acad. Sci. USA, 1996, 93, 11652-11657). Furthermore, mutagenesis experiments have pointed out the 22nd amino acid position in the MscL channel as one of the important sites that affect the channel gating properties and kinetics. While a hydrophobic moiety at this position makes the channel constriction more stable, a hydrophilic
10 residue destabilises this region and leads to channel opening without any extra energy input (Yoshimura, K., *et al.*, Biophys. J. 1999, 77, 1960- 1972). Thus, in a preferred embodiment of the invention, a protein channel is modified with a compound of the invention at the position that corresponds to residue 22 of the *E.coli* MscL protein. In case of a preferred *E.coli* MscL, the original amino acid at the 22nd
15 position, glycine, is mutated to a cysteine in order to create a target site for the attachment of pH-sensitive compound. Recently, Bartlett *et al.* identified new amino acid residues in the transmembrane domain of MscL that can be modified with MTSET, a positively charged MTS reagent, and gate the channel even in iso-osmotic conditions in intact *E. coli* cells (Bartlett, *et al.*, PNAS 2004, 101, 10161-10165).
20 These residues can be modified with a compound of the invention. Combinations of different target residues for attachment of compounds that are pH-sensitive, light-sensitive or a combination of both can also be used to control functional properties of the channel protein. Alternatively, target residues for attachment can be combined with other mutations that modify channel function.
25 These can be located either closely together in the primary sequence or in different regions of the polypeptide, or even in different polypeptide chains of the multimeric channel protein. The invention further provides a modified mechanosensitive channel protein wherein said channel protein is modified with at least one compound of the invention. In a preferred embodiment, said modified mechanosensitive channel
30 protein is the MscL channel protein of *E. coli*, or a functional equivalent thereof. As said, a compound can be straightforwardly attached to a desired target residue, optionally via cysteine modification, by contacting the protein with the compound under conditions that allow the formation of a covalent bond between said residue and

group C of said compound, preferably followed by removal of excess compound (see Example 2). Modification of the channel protein with a compound of the invention can be used to modify the gating properties of a proteinaceous valve. More specifically, the compounds allow to convert a channel which was previously only mechanosensitive
5 into a pH- and/ or light-responsive channel. In some cases, in addition to the pH- and/ or light-responsive gating, the mechanosensitivity of the channel protein is retained. A modified channel protein according to the invention is highly attractive for use as a triggerable valve in drug delivery vehicles, e.g. for controlled and/or localised release of therapeutic molecules at a target site. A modified protein channel can be
10 reconstituted in a lipid-based delivery vehicle such as proteoliposomes, which can subsequently be loaded with one or more therapeutic molecules. It has been found that MscL is active in lipid vehicles that consist of positively and/or neutrally charged lipids as well as negatively and/or neutrally charged lipids. Lipid vehicles comprising said positively and/or neutrally charged lipids are more resistant to uptake by cells of
15 the mononuclear phagocytic system. Lipid vehicles of the invention therefore preferably comprise positively and/or neutrally charged lipids. Such vehicles exhibit improved half-lives in the bloodstream. Such vehicles also demonstrate improved targeting to non-mononuclear phagocytic system cells. The lipid part directed towards the exterior of a lipid vehicle of the invention preferably consists predominantly of
20 positively and/or neutrally charged lipids, thereby postponing or nearly completely avoiding cellular uptake through negatively charged lipids. Apart from increasing the half-life of the vehicle in the blood stream, different lipids can also be used to alter the amount of added pressure needed to activate the channel in the vehicle. This results from changes in the lateral pressure profile across the membrane associated with
25 alterations in membrane composition.

In another preferred embodiment, the lipid vehicle contains neutral lipids as well as lipids with a polymer chain attached to the polar headgroup, preferably polyethyleneglycol (PEG), more preferably with a molecular weight (of the PEG chain) of approximately 2000. Although these liposomes may contain negative charge, they
30 have been shown to have an increased circulation time in blood (see e.g. Drummond, D. C., *et al.*, Pharmacol. Rev. 1999, 51, 691-744 and references therein) compared to liposomes that do not expose polymer chains on their surface. It is believed that this results from the fact that the polymer chains exclude (at least partially) other

macromolecules from the exterior of the liposome, reducing in this way binding of blood plasma opsonins, and consequently, uptake by the reticuloendothelial system.

Therapeutic molecules are typically biologically active compounds or precursors thereof, such as pro-drugs which are converted in the body into an active drug. They include anti-neoplastic agents, microbial compounds, immunomodulators, peptides and peptidomimetics. A biologically active compound may be included in the delivery vehicle as a single active agent or in combination with other biologically active compounds.

A delivery vehicle according to the invention may also comprise targeting ligands to achieve site-specific targeting of the vehicle to a predetermined target site. Depending on the target site, various types of targeting ligands may be attached to the liposome surface, including (anti-tumour) antibodies or fragments thereof, carbohydrates and vitamins.

The application of a modified protein channel as a valve in proteoliposomes that can be triggered is nicely illustrated by a fluorescent efflux assay described in Example 3. Herewith, a method is provided for controlling the release of therapeutic molecules at a target site of a subject, comprising loading a delivery vehicle comprising a modified channel protein, such as MscL or a functional equivalent thereof, with one or more drugs, introducing the loaded delivery vehicle into the subject to allow delivery to the target site and triggering release of the drug(s) by a physiological trigger (pH below appr. 7.4), optionally following an external trigger (illumination with light). A pharmaceutical composition comprising a delivery vehicle with a modified MscL protein of the invention and a pharmaceutically acceptable carrier is also provided. A modified channel protein or a delivery vehicle comprising a channel protein is of use for the (time) controlled release of a therapeutic molecule to a target site. For instance, it is known that in tumours the pH is very often considerably lower than in the normal tissue surrounding the tumour. Other areas in the body that have a lowered pH are the liver, areas of inflammation and ischemic areas. By using a pH-sensitive delivery vehicle of the invention, it is now possible to safely deliver an anti-cancer drug in an encapsulated form to a tumour-site, where its release is subsequently triggered by the locally lowered pH, which release may be further controlled by an external light stimulus. Various anti-cancer drugs may be

used, including cisplatin, carboplatin, methotrexate, 1- β -D-arabino-furanyl-cytosine (ara-C), 5-fluoro-uracil, floxuridine, and gemcitabine.

For an ideal triggered drug release system, the compound-modified protein channel molecule in the liposomes should be sensitive enough to respond to the small changes of stimuli. The pH in solid tumours and at sites of inflammation is about 0.4-0.8 pH unit lower than physiological pH (pH 7.3-7.5). We herein provide a modified channel protein that is highly pH-sensitive; when this channel protein was reconstituted in proteoliposomes a decrease of only 0.2 pH units resulted in up to 30 % more channel activity. This pH range of activity is also compatible with physiological conditions.

In addition to the sensitivity, the response time to the trigger may also be important. For instance in endosome targeted delivery, the liposomes should respond to the pH of endosomes within 10-30 minutes before going to the lysosomal degradation route. The modified channel proteins according to the invention respond to pH changes almost immediately. Incorporation of a photocleavable group in a compound of the invention provides a control mechanism over the response time of the compound such that it can be decided when to activate the compounds by removal of the photolysable group. The modified channel proteins of the invention do not negatively affect the integrity and stability of the drug delivery vehicle.

Figure Legends

Figure 1. The compounds of the invention are composed of different groups. Compounds comprise a coupling group C in combination with either a chargeable group Q or (in case of the light sensitive compounds) with a photocleavable chargeable group P. Photocleavage of group P leads to unmasking of a chargeable moiety. The chargeable group Q or chargeable moiety of group P released upon illumination can either be in a charged or uncharged state depending on the environment or external stimuli and will be directly responsible for alternation of the protein properties. The coupling group C can bind to a cysteine residue of the protein, thereby attaching the compound to the channel protein. The Q or P group may be connected to a C group through a linker L. The Q groups are designed to achieve a suitable pKa of the

charged group. Me = methyl; Et= ethyl; Pr=propyl; iPr= isopropyl; Ph=phenyl;
Bn=benzyl;

Figure 2. The general chemical modification mechanism of a cysteine sulfhydryl of a
5 protein (e.g. MscL channel protein) with methylthiosulfonate (MTS)-based compounds
i.e. those comprising C₃ as coupling group. Methylthiosulfonate reacts very rapidly
and specifically with cysteine sulphydryls and attaches the compound to the protein.
The sulfinic acid by-product of the reaction decomposes rapidly to low-molecular-
weight volatile products, which do not, in general, affect the stability of the disulfide
10 bond formed, or the activity of the channel protein.

Figure 3. The photocleavage reaction of compound D: P₁[R₃= OMe, R₁= H]-L₁[n= 1]-C₃
Figure 4. Schematic presentation of the synthesis of a pH-responsive compound
wherein the coupling group is based on MTS (compound Q₁-L₁-C₃). (1): Glycine or its
15 N alkylated derivative; (2): 2-bromo-ethyl ester of glycine or its N alkylated
derivative; (3): 2-methanesulfonylsulfanyl-ethyl ester of glycine or its N alkylated
derivative.

Figure 5. Examples of pH-sensitive compounds of the formula Q₁-L₁-C₃. A) R₁ and R₂
20 are both H (compound A: Q₁[R₁=R₂=H]-L₁[n= 2]-C₃). B) R₁ is H and R₂ is CH₃
(compound B: Q₁[R₁= H, R₂=Me]-L₁[n= 2]-C₃). C) Both R₁ and R₂ are CH₃ (compound
C: Q₁[R₁=R₂=Me]-L₁[n= 2]-C₃).

Figure 6. Schematic presentation of the synthesis of a light-sensitive pH-responsive
25 compound of the formula P₁[R₃= OMe, R₁= H]-L₁[n= 1]-C₃ (compound D). (1): 2-
bromoethylamine hydrobromide; (2): Methanethiosulfonic acid S-(2-amino-ethyl) ester
hydrobromide; (3): Methanethiosulfonic acid S-[2-(4,5-dimethoxy-2-nitro-
benzyloxycarbonylamino)-ethyl] ester.

30 Figure 7. Calcein Efflux Assay A) Calcein containing proteoliposomes were prepared
and liposomal fraction was separated from the free calcein by a Sephadex G50 size
exclusion column; B) The schematic presentation of the efflux ; C) An example of an
activity measurement in the efflux assay. Arrow indicates the time when the trigger is

applied (In this particular case the trigger is the application of 0.8 mM final concentration of [2-(Trimethylammonium)-ethyl]-methanethiosulfonate bromide (MTSET) into the measurement cuvette containing proteoliposomes).

- 5 Figure 8. Calcein efflux assay using proteoliposomes containing the MscL channel protein modified with the pH-responsive compound A ($Q_1[R_1=R_2=H]-L_1[n=2]-C_3$). The efflux assay was performed at different pH's. A) Channel activity at different pH's; B) the maximum % fluorescence is shown as a function of pH.
- 10 Figure 9. Calcein efflux assay using proteoliposomes containing the MscL channel protein modified with the pH-responsive compound B ($Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$). The efflux assay was performed at different pH's. A) Channel activity at different pH's; B) the net % fluorescence is shown as a function of pH.
- 15 Figure 10. Calcein efflux assay using proteoliposomes containing the MscL channel protein modified with the pH-responsive compound C ($Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$). The efflux assay was performed at different pH's. A) Channel activity at different pH's; B) the maximum % fluorescence is shown as a function of pH.
- 20 Figure 11. Calcein efflux assay using proteoliposomes containing the MscL channel protein modified with the light-sensitive pH-responsive compound D ($P_1[R_3=OMe, R_1=H]-L_1[n=1]-C_3$). Calcein efflux assay of liposomes containing the modified protein is performed at different pH's. A) Channel activity at different pH's after initial illumination (+) or no illumination (-); B) The maximum % fluorescence is shown as a
- 25 function of pH.

Figure 12. Chemical synthesis of light-responsive compound $P_6-C_1[X=I]$ (*compound E*).

- 30 Figure 13. Patch Clamp analysis of pH-responsive compounds. In all the cases, the upper trace shows the current and the lower one shows the negative pressure. The dashed lines indicate the value "0" for the particular signal. The channel openings are seen as boxes or spikes in the current signal. The numbers (0-3) indicates the number

of MscL channels. A) A trace of unmodified MscL gating in response to the negative pressure. B) Patch clamp recording of compound B ($Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$)-modified MscL at pH 7.2. C) Patch clamp recording of compound B-modified MscL at pH 6.2. D) Patch clamp recording of compound C ($Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$)-modified MscL at pH 6.3.

Figure 14. Light induced gating of MscL modified with the light-responsive compound E ($P_6-C_1[X=I]$). A) The mechanism of charge generation (in this case a negative charge). B) Calcein efflux assay of the compound modified MscL. C) Patch clamp recording of the modified MscL at dark. D) Patch clamp recording of the modified MscL after 10 minute illumination with 366 nm light.

Figure 15. Light-sensitive pH-responsive activity of MscL modified with compound F ($P_2[R_3=OMe, R_1=Me]-L_1[n=2]-C_3$). A) The mechanism of charge generation in response to light and pH. B) Calcein efflux assay of the modified MscL.

Figure 16. The activity of double cysteine MscL mutant modified with two compounds C per subunit of MscL. A) The schematic presentation of a modified MscL. B) Calcein efflux assay of the modified MscL. Error bars indicate the standard deviation of two independent experiments.

Examples

Example 1. Synthesis of pH- and light-responsive compounds

Methylthiosulfonate (MTS) derivatives react very rapidly and specifically with cysteine sulphydryls and introduce the functional group into the protein. The sulfinic acid by-product of the reaction decomposes rapidly to low-molecular-weight volatile products, which do not, in general, affect the stability of the disulfide bond formed, or the activity of the enzyme. This example shows the synthesis of both pH and light sensitive MTS-based compounds.

1. A. General Synthesis of the pH-responsive compounds

Material and Methods

The schematic presentation of the compound synthesis is given in Figure 4. In the first reaction glycine or its N alkylated derivative (1) in the form of the free compound or as a HCl salt (20 mmol) was suspended in 2-bromoethanol (14.3 ml, 200mmol) and cooled to 0°C. Thionyl chloride (1.8 ml, 25 mmol) was added dropwise and reaction mixture was stirred at room temperature until a clear solution was obtained. Resulting solution was poured into 200 ml of ether, precipitated solid was filtered, washed with ether and dried in vacuo. In the case that only oil separates instead of precipitate, ether was decanted; oil was washed with ether (2x 100ml) and all the residual solvents were removed in vacuo. Oil usually solidifies upon standing overnight at 4°C. Compound bromo-ethyl ester of glycine or its N alkylated derivative (2), was obtained as HCl salt.

In the second reaction the salt of 2-bromo-ethyl ester of glycine or its N alkylated derivative (2) (10 mmol) was dissolved in DMF (10 ml) and sodium methanethiosulfonate (1.47g, 11 mmol) was added. Mixture was heated at 70°C for 4 hours, solid precipitate was filtered out and DMF was evaporated in vacuo. The residue was dissolved in small amount of boiling acetonitrile (20 ml), filtered and filtrate evaporated in vacuo. Product, 2-methanesulfonylsulfanyl-ethyl ester of glycine or its N alkylated derivative (3), is also obtained as HCl salt. Solid products can be recrystallized from acetonitrile-ether or ethanol-ether.

Results and Discussion

The yield of the first synthesis reaction was over 95%, and the purity of the compound, 2-bromo-ethyl ester of glycine or its N alkylated derivative (2), was over 98%. The yield of the second reaction was 70 – 90%. The resulted compound, 2-methanesulfonylsulfanyl-ethyl ester of glycine or its N alkylated derivative (3), may contain small amount (<5%) NaCl.

1.A.1. The first pH responsive compound obtained by a starting compound in which both two functional groups (R_1 and R_2) of the Glycine or its N alkylated derivative (1) were hydrogen. The synthesized molecule (compound A: $Q_1[R_1=R_2=H]-L_1[n=2]-C_3$) is shown in Figure 5A.

1.A.2. The second pH compound obtained by a starting compound in which the functional groups (R_1 and R_2) of the Glycine or its N alkylated derivative (1) were a hydrogen and a methyl, respectively. The synthesized molecule (compound B: $Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$) is shown in Figure 5B.

5

1.A.3. The third pH compound obtained by a starting compound in which both functional groups (R_1 and R_2) of the Glycine or its N alkylated derivative (1) were methyls. The synthesized molecule (compound C: $Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$) is shown in Figure 5C.

10

1. B. General Synthesis of Light- Sensitive pH-responsive Compounds

Material and Methods

The schematic presentation of the synthesis of a light sensitive compounds is given in Figure 6. In the first reaction sodium methanethiosulfonate (1.47g, 11 mmol) was added to the solution of 2-bromoethylamine hydrobromide (1) (2.05g, 10 mmol) in DMF (5 ml) and mixture was heated at 70°C for 4 hours, solid precipitate was filtered out and DMF was evaporated in vacuo. The residue was dissolved in a small amount of boiling acetonitrile (20 ml), filtered and the filtrate evaporated in vacuo. The recrystallization from acetonitrile-ether gave product.

20

In the second reaction, 236 mg, 1 mmol of methanethiosulfonic acid S- (2-amino-ethyl) ester hydrobromide (2) was dissolved in 3 ml of DMF and 6-nitroveratrylchlorofotmate (303 mg, 1.1 mmol) and pyridine (0.5 ml) were added. After overnight stirring, the mixture was poured into water (10 ml) and extracted with dichloromethane (3x 30 ml). All the solvents were evaporated in vacuo and product, methanethiosulfonic acid S-[2-(4,5-dimethoxy-2-nitro-benzyloxycarbonylamino)-ethyl] ester (3; compound D; $P_1[R_3=OMe, R_1=H]-L_1[n=1]-C_3$), chromatographed on Si-gel using dichloromethane as an eluent.

25

Results and Discussion

30

The yield of the first reaction was 85%, that of the second reaction was 48%. The product (3; compound D; $P_1[R_3=OMe, R_1=H]-L_1[n=1]-C_3$) was soluble in DMF and DMSO, was a little soluble in dichloromethane and chloroform and was very badly soluble in other common solvents.

Example 2. Chemical modification of the MscL Channel Protein

5

All synthesized compounds were attached to the MscL protein from *E. coli* via cysteine modification. In this example, the 22nd position of the protein was mutated to a cysteine.

10 *Material and Methods*

A membrane pellet fraction was obtained from a fermentor culture of *E.coli* PB104 cells containing the plasmid pB104 carrying the MscL(G22C)-6His construct. MscL, was isolated to near homogeneity via a single nickel-nitriloacetic acid (Ni-NTA) metal-affinity chromatography step.

15-

1 volume of isolated, detergent solubilized MscL (0.3 mg/ml) was incubated with 0.5 volume of 160 mM compound (A-C) for 15 minute at room temperature. The protein modification reaction was stopped and the excess label was removed by applying the sample into a gel filtration column. The protein concentration of elution fractions was
20 determined by Bradford Assay.

The modified protein was reconstituted in synthetic lipid membranes in order to follow the channel activity. For this purpose, DOPC: Cholesterol: DSPE-PEG (70:20:10 Molar ratio) liposomes were titrated with Triton- X100 until saturation.

25 Titrated liposomes were mixed with modified protein in 1:120 (wt: wt) protein to lipid ratio. Biobeads were used to remove the detergent. A self-quenching dye, calcein, was added and included into the interior of the proteoliposomes during reconstitution.

Results and Discussion

30 5-8 g wet weight membrane pellet was obtained from a 10 liter fermentor culture of *E.coli* PB104 cells. Approximately 3 mg protein/l of fermentor culture was isolated to near homogeneity via a single metal-affinity chromatography step. The isolated detergent soluble protein was stable at least 6 months at -80 °C. The efficiency of

protein modification with the compound was monitored by both a calcein assay and by mass spectroscopy.

5 **Example 3. Functionality of a modified MscL Channel Protein**

Proteoliposomes containing modified MscL protein were analyzed by a fluorescent dye efflux assay. The activity of the channel was measured as an increase in the fluorescence of a reporter molecule, the dye calcein. Calcein is included in the
10 liposome interior at a self quenching concentration, but when the calcein escapes into the exterior medium the dilution is such that quenching is completely abolished.

Materials and Methods

The schematic presentation of the efflux assay is given in Figure 7. During
15 reconstitution of the protein into the mixture of synthetic lipids in the presence of a detergent, a self quenching dye, calcein, is also added in to the mixture to a final concentration of 50 mM. After the reconstitution is completed by the removal of the detergent, the calcein containing liposomal fraction was separated from the unencapsulated free calcein by passing the sample through a Sephadex G50 size
20 exclusion column (Figure 7A).

A portion of the liposomal fraction is placed in 2 ml iso-osmotic buffer and the activity of the channels is followed by measuring the increase in fluorescence as calcein becomes dequenched upon being released through the channel protein in response to a
25 stimulus (Figure 7B). Fluorescence was monitored with a SLM 500 spectrofluorometer. Excitation and emission wavelengths were 490 (slit 2 nm) and 520 nm (slit 4 nm), respectively. A positively charged MTS reagent (MTSET) was used as a positive control. Since MTSET always carries a positive charge, it always opens the channel after it has reacted with MscL at the free sulfhydryl residue at the 22nd position
30 (Figure 7C).

Results and Discussion

The calcein efflux assay gives an opportunity to follow the activity of MscL channels in model drug containing proteoliposome ensembles in response to an applied stimulus. The system works fast and gives reproducible results.

5

Example 4. A. Channel activity of the protein modified with a pH-responsive compound

The MscL protein was modified with the different pH-responsive compounds. The activity of the channel in response to pH was analyzed by the calcein efflux assay. In order to do that the same buffer at different pH's were prepared and the same sample is analyzed in each buffer.

Example 4. A. 1. Compound A: $Q_1[R_1=R_2=H]-L_1[n=2]-C_3$
 The first pH compound has $-CO(O)-CH_2NH_2$ ($Q_1 [R_1=R_2=H]$) as a chargeable group Q. The activity of proteins modified with compound A ($Q_1[R_1=R_2=H]-L_1[n=2]-C_3$) is shown in Figure 8. The modified protein showed a pH dependent activation, especially at lower pH's although there was some activity at physiological pH.

Example 4. A. 2. Compound B: $Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$

The second pH compound has $-CO(O)-CH_2NHCH_3$ ($Q_1[R_1=H, R_2=Me]$) as a chargeable group Q. The activity of proteins modified with compound B ($Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$) is shown in Figure 9. The modified protein showed a pH dependent activation. Its pKa is approximately 8. This particular compound started to show channel activity below pH 8.

Example 4. A. 3. Compound C: $Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$

The third pH compound has $-CO(O)-CH_2N(CH_3)_2$ ($Q_1[R_1=R_2=Me]$) as a chargeable group Q. The activity of proteins modified with compound C ($Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$) is shown in Figure 10. The modified protein had no activity at pH 7.5, and started to be active at around pH 7.03. This is an ideal compound for the drug delivery to a target site at which the pH is lower than physiological pH. The modified channel will

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stay close at the normal body pH of 7.4 but will be activated (opened) at the low pH of the target site.

Example 4. B. Channel Activity of the protein modified with a light- sensitive compound.

The MscL protein was modified with a light sensitive compound. The activity of the channel was analyzed in response to pH. After measuring the initial fluorescence value for samples at each pH, they were exposed to 366 nm light for 5-10 minutes.

Then the measurement was continued as before. Results are given in Figure 11.

The compound stayed inactive until the photolysis occurs. After the cleavage of the light- sensitive part by illumination, the chargeable group left behind showed a pH dependent activity. The light sensitivity can be used both as a separate control tool to open the channel but also as a masking group to protect the chargeable group until the time a response (i.e. release) is desired.

Example 5. Chemical synthesis of a light –responsive compound

This example shows the synthesis of a light responsive compound (compound E: $P_6-C_1[X=I]$) as shown in Figure 12.

Example 5. A. 5-dimethoxy-2-nitrobenzyl 2-bromoacetate

To the solution of 6-nitroveratryl alcohol (0.43g, 2 mmol), Et_3N (0.56 ml, 4 mmol) and DMAP (24 mg, 0.2 mmol) in $CHCl_3$ (5 ml) was slowly added bromoacetyl bromide (0.19 ml, 2.2 mmol). After stirring at room temperature for 5 h CH_2Cl_2 (20 ml) was added and mixture was washed with HCl (1M, 2x10 ml), $NaHCO_3$ (sat., 10 ml) and water (10 ml). Organic phase was dried over Na_2SO_4 , evaporated and purified by chromatography (Si-gel, CH_2Cl_2) to yield 4,5-dimethoxy-2-nitrobenzyl 2-bromoacetate (368 mg, 55%).

Example 5. B. 4,5-dimethoxy-2-nitrobenzyl 2-iodoacetate

A 4.5-dimethoxy-2-nitrobenzyl 2-bromoacetate (100 mg, 0.3 mmol) was dissolved in acetone (2 ml) and NaI (50 mg, 0.33 mmol) was added. After stirring for 2h at room temperature mixture was filtered, evaporated and purified by chromatography (Si-gel, CH₂Cl₂) to yield 4.5-dimethoxy-2-nitrobenzyl 2-iodoacetate (compound E: P₆-C₁[X= I]) (97 mg, 85%).

Example 6. Single molecule level analyses of MscL channel proteins modified with a pH-responsive compound

10

The activity of the chemically modified MscL is analyzed real time at single molecule level with patch clamp, a classical electrophysiological technique. This technique allows to seal a patch of membrane, which has embedded channel proteins in it, into the tip of a glass micropipette, and to monitor the ionic current flowing through the open channels in response to different conditions.

15

As a mechanosensitive channel, unmodified MscL needs significant amount of tension on the membrane to gate. In patch clamp conditions it corresponds to the application of negative pressure almost as high as that causes rupturing of the patched membrane. On the other hand, the presence of charge in the pore region of the protein reduces this need significantly, and channel protein starts to gate even without any tension.

20

This example shows the patch clamping of modified channel proteins at different pH's with or without applied negative pressure. The channel activity was measured by following its conductance.

25

Materials and Methods

The protein from cysteine mutant MscL (cysteine in its 22nd amino acid position) was isolated, modified and reconstituted as explained in Example 2. After reconstitution, giant proteoliposomes were prepared as indicated (Delcour, A. H., Martinac, B., Blattner, F. R., Kung, C., Biophys. J.56, 1989, 631-636). 10 µl rehydrated lipids were placed in 100 µl bath solution and resulting giant liposomes were patched by using

30

glass pipettes with 1 μm tip size. They were made from 100 μl borosilicate capillaries in Sutter 97/IVF micropipette puller. Single channel recordings performed at +20 mV in symmetrical buffer conditions in 200 mM KCl, 100 mM MgCl_2 , 5 mM HEPES, pH 6-8. Data were amplified and filtered in Axopatch1D amplifier and sampled in
5 Digidata 1322A digitizer. Recordings were analyzed with pCLAMP8 software.

Example 6. A. Compound B; $\text{Q}_1[\text{R}_1=\text{H}, \text{R}_2=\text{Me}]-\text{L}_1[\text{n}=2]-\text{C}_3$

A schematic presentation of unmodified MscL activity in patch clamp analyses is given in Figure 13 A. It shows single channel openings in response to the applied
10 negative pressure to the patch pipette. When there is no pressure, no current can pass through the channel but as soon as pressure is applied, then channels open and the characteristic ionic current can be measured.

The patches containing the channel protein, which was modified with a pH-
15 responsive compound "compound B ($\text{Q}_1[\text{R}_1=\text{H}, \text{R}_2=\text{Me}]-\text{L}_1[\text{n}=2]-\text{C}_3$)" were analyzed at two pH's. As shown in Figure 13 B the channel didn't give any activity at pH 7.2 under the patch clamp conditions ($\text{n}=10$) even when negative pressure was applied to the membrane until the patched membrane ruptured (the mean negative pressure was $79 \text{ mmHg} \pm 27 \text{ mmHg}$). At this pH, the label is mainly in its neutral form.

20

On the other hand, at pH 6.2, below the pK_a of the pH-responsive compound, where the compound is mainly in its protonated form, channel gated even without any applied negative pressure ($\text{n}=4$) (Figure 13 C).

25 Example 6. B. Compound C: $\text{Q}_1[\text{R}_1=\text{R}_2=\text{Me}]-\text{L}_1[\text{n}=2]-\text{C}_3$

The channel protein, which was modified with a pH- responsive compound "compound C ($\text{Q}_1[\text{R}_1=\text{R}_2=\text{Me}]-\text{L}_1[\text{n}=2]-\text{C}_3$)" was analyzed at different pH's. Figure 13 D shows the pH dependent spontaneous gating of the modified channel at pH 6.3. The channel gated only at low pH environment.

30

Results and Discussion

In experiments, at high pH's the modified channel protein behaved as unmodified one because the pH- responsive compounds were mainly in their neutral form. The

channels also required very high negative pressure on the membrane in order to gate at high pH if they did at all. Most of the time it was so high that the patch membrane bursted before seeing any channel activity. On the other hand, at low pH's as soon as compounds became charged, due to their location within the channel protein, the
5 channels gated simultaneously without any tension.

The results of this example are in good agreement with that of Example 4.A.2. and 4.A.3. In those cases channels, which were modified with compound B ($Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$) and compound C ($Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$), respectively, were
10 analyzed in calcein containing liposomes in the calcein efflux assay.

Example 7. Gating of the mechanosensitive channel protein, MscL, in response to light

15

This example shows the modification of a mechanosensitive channel protein MscL into a light sensitive channel. The light-sensitive compound of the formula P_6-C_1 (compound E: $P_6-C_1[X=I]$) is designed to stay uncharged until being illuminated with 366 nm wavelength light. After modification of the channel protein with the
20 compound, illumination of the sample lyses the photocleavable part of the compound and leaves a negative charge attached to the protein in its hydrophobic constriction site (Figure 14 A). Again, generation of charge within the pore region of the channel leads spontaneous channel gating.

25 *Materials and Methods*

The protein from cysteine mutant MscL (cysteine in its 22nd amino acid position) was isolated and modified as explained in Example 2, except that the light-responsive compound E ($P_6-C_1[X=I]$) was dissolved in DMSO and incubated with the protein at a 1:500 molar ratio (protein: compound) for 45 minutes.

30

The resulted modified channel was reconstituted in the presence of a fluorescent dye as explained in Example 3 for the calcein efflux experiment. Another sample of the

modified channel was prepared for patch clamp experiments as indicated in Example 6.

Example 7. A. Calcein Efflux Assay

5 After modification of the channel protein with the light-responsive compound E (P₆-C₁[X= I]), photolysis of the compound was followed with UV-Vis absorption spectroscopy. The disappearance of the band centered at 346 nm and appearance of a band at 374 nm indicated the photolysis of the compound and generation of acid and 6-nitrosoveratryl aldehyde.

10

In the calcein efflux assay, proteoliposomes containing the chemically modified channel protein were tested for the release of the fluorescence dye in the dark or after illumination. In the dark, the control sample did not give any release because the photocleavable part stayed intact and thus had no gating power on the channel. The
15 duplicates of the same sample, on the other hand, released the fluorescent dye as soon as proteoliposomes were exposed to ultraviolet light. In this case, the photocleavable moiety of the P group was released to leave a negative charge attached to the protein in the charge sensitive hydrophobic core. This resulted in channel gating in isoosmotic conditions (Figure 14 B).

20

Example 7. B. Patch clamp analyses

Membranes having modified channels were patched at dark or 5 to 10 minutes after illumination with 366 nm light. Figure 14 C shows that there was no channel gating at dark. Even application of a negative pressure up to membrane rupture, channels
25 did not gate. On the other hand, channels gated simultaneously with no tension after illumination (Figure 14 D).

Results and Discussions

As can be seen in figure 13 A, an unmodified channel protein requires tension on the
30 membrane for gating. In this example we modified the channel in a way that it gated in response to the light even if there was no tension.

In this particular case a chargeable moiety was protected with a photocleavable moiety until illumination with UV light. The light treatment cleaved the photolysable part of the compound and left behind a negatively charged compound attached to the protein pore region. Again the charges in the otherwise hydrophobic pore region lead to spontaneous channel gating.

In both calcein efflux assay and patch clamp experiments we showed that the modified MscL channel protein modified with this compound gates in response to light.

Example 8. Light-sensitive pH-responsive activity of MscL

A compound of the formula $P_2-L_1-C_3$ (compound F: $P_2[R_3=OMe, R_1=Me]-L_1[n=2]-C_3$) was synthesized and coupled to the protein. Illumination of the modified protein with 366 nm light cleaves off a part of the P_2 group to generate "compound B ($Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$)" in its protein attached form. Then, if the pH of the environment is high, only a small portion of the label has charge and there is not so much channel activity. On the other hand, if the pH of the environment is low after the photolysis of the light sensitive part, then, compound B ($Q_1[R_1=H, R_2=Me]-L_1[n=2]-C_3$) is mainly in a protonated form and channels start to gate spontaneously. However, for channel to gate even at low pH, the first step is the cleavage of the light-sensitive protection part. This property gives an opportunity to control the time of channel activation.

Materials and Methods

The protein was isolated and modified as in Example 2 and reconstituted in the presence of calcein as indicated in Example 3. The resulting liposomes were tested at different pH's at dark or after illumination with 366 nm light.

Results and Discussion

The structure of the light-sensitive pH-responsive compound F ($P_2[R_3=OMe, R_1=Me]-L_1[n=2]-C_3$) is given in Figure 15 A.

The modified and reconstituted protein was analyzed in calcein efflux assay. 5 sets of the same sample were analyzed at pH 5.7 and pH 7.2. All samples were measured for their initial fluorescence and then one group was left at dark and the duplicate was illuminated with 366 nm light for 10 min. The pH of one of the samples was
5 immediately changed from 5.7 to pH 7.2 and the fluorescence of each sample was followed in time.

As can be seen in Figure 15 B, samples left at dark didn't release any fluorescent dye independent of the pH (samples marked with the cross and the plus). On the other
10 hand, if samples were illuminated, the one at low pH (solid diamonds) gave a much higher efflux than the one at high pH (solid triangles). When the last sample was first illuminated at pH 5.7 and immediately experienced pH 7.2 (open diamonds), it gave almost the same initial kinetics as the sample which was illuminated at pH 5.7. But after the point where the buffer pH was increased, the efflux stopped.

15 The results clearly indicates a light protection of an otherwise pH-responsive compound. The new compound provides a tool to control the time of activation of the channel.

20

Example 8. New targets on MscL channel protein for the chemical modification: Combination of the 22nd and 23rd amino acid positions

In order to further improve modulation of the activity of chemically modified MscL, in
25 addition to the amino acid at the 22nd position of the channel, the 23rd position is also replaced with cysteine. This way if all the subunits are modified, one can introduce two times more charge.

This example compares the single and double cysteine mutants for their performance
30 in efflux from proteoliposomes.

Materials and Methods

The protein from each mutant was obtained and modified with the Compound C ($Q_1[R_1=R_2=Me]-L_1[n=2]-C_3$) as explained in Example 2 and the reconstitution for calcein efflux assay was as in Example 3.

5 *Results and Discussion*

The schematic presentation of the modified double mutant is shown in Figure 16 A. It shows one subunit of MscL out of 5 .

10 The modification of the single and double mutant was confirmed with the mass spectrometry.

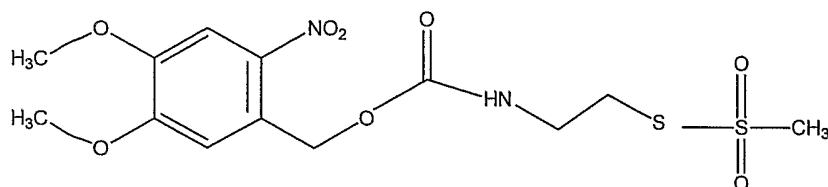
As shown in Figure 16 B, the double mutant (solid squares) gave a better release at low pH than the single mutant (solid diamonds). (Results were from two separate experiments). Both modified channels didn't release the liposomal content at high pH.

15 -

This example shows that we can combine different target positions in the protein and control the channel gating.

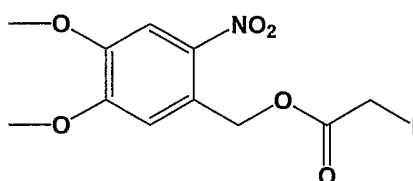
Claims

1. A protein modified with a pH-responsive or light responsive compound of the general formula Q-C or P-C, wherein Q is a chargeable group selected from the groups Q₁, Q₂, and Q₃, wherein P is a photocleavable chargeable group selected from the groups P₁, P₂, P₃, P₄, P₅, P₆, P₇ and P₈ and wherein C is a coupling group selected from the groups C₁, C₂, C₃, C₄, C₅ and C₆ as depicted in Figure 1.
2. Modified protein according to claim 1, wherein group Q or group P is connected to group C via a linker group L selected from the linker groups L₁, L₂, L₃ and L₄ as depicted in Figure 1.
3. Modified protein according to claim 1 or 2, wherein said protein is a mechanosensitive channel protein, preferably MscL channel protein of *E.coli*, or a functional equivalent thereof.
4. A pH-responsive or light responsive compound capable of controlling the channel activity of a mechanosensitive channel protein, preferably MscL channel protein of *E.coli*, or a functional equivalent thereof, said compound of the general formula Q-C or P-C, wherein Q is a chargeable group selected from the chargeable groups Q₁, Q₂, and Q₃ wherein P is selected from the photocleavable chargeable groups P₁, P₂, P₃, P₄, P₅, P₆, P₇ and P₈ and wherein C consisting of a coupling group selected from the coupling groups C₁, C₂, C₃, C₄, C₅ and C₆ as depicted in Figure 1.
5. A compound according to claim 4, wherein group Q or group P is connected to group C via a linker group L selected from the group comprising the linker groups L₁, L₂, L₃ and L₄ shown in Figure 1.
6. pH-responsive compound of the formula R₁R₂N-CH₂-(CO)O-(CH₂)₂-S(SO₂)-CH₃, wherein R₁ and R₂ are each independently H or CH₃, preferably wherein R₁ and R₂ are CH₃.
7. Light-sensitive pH-responsive compound of the formula



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8. Light-responsive compound of the formula



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9. Method for providing a modified protein, preferably a mechanosensitive channel protein, more preferably the MscL channel protein from *E.coli*, or a functional equivalent thereof, comprising the chemical modification of the protein with a compound according to any one of claims 4 to 8 by the covalent attachment of said compound to a target residue of said protein, preferably a cysteine residue, and wherein said target residue is located in a region of said protein which upon introduction of a charge and/or polar group modulates the functionality of said protein.

10. Method according to claim 9, wherein said target residue is introduced into said protein using mutagenesis.

11. Method according to claim 9 or 10, wherein said protein is a mechanosensitive channel protein, preferably MscL channel protein of *E.coli* or a functional equivalent thereof, and wherein introduction of a charge and/or polar group induces opening of said channel.

12. Method according to claim 11, wherein introduction of a positive charge induces opening of the channel.

13. Method according to claim 11 or 12, wherein said target residue is located in a region that corresponds to amino acid residues 1-14, residues 15-45, residues 46-75 or residues 76-100 of the MscL protein of *E.coli*.

5 14. Method according to claim 13, wherein said target residue is located in the region that corresponds to the region around position 23 of the MscL protein of *E.coli*.

15. Method according to claim 14, wherein said target residue corresponds to amino acid residue 22.

10

16. A delivery vehicle for controlled and/or localised release of therapeutic molecules, preferably a lipid-based delivery vehicle, comprising a modified channel protein according to claim 3 or obtainable by a method according to any one of claims 11 to 15.

15

17. Delivery vehicle according to claim 16 comprising a therapeutic molecule.

18. A pharmaceutical composition comprising a delivery vehicle according to claim 14 or 15.

20

19. A method for controlling the release of a therapeutic molecule at a target site of a subject, comprising loading a delivery vehicle according to claim 16 with a therapeutic molecule, introducing the loaded delivery vehicle into the subject to allow delivery to the target site and triggering the release of said therapeutic molecules.

25

20. A method according to claim 19, wherein said triggering comprises exposure to a low pH, optionally preceded by illumination.

30

21. Use of a modified channel protein according to claim 3 or obtainable by a method according to any one of claims 11 to 15, or a delivery vehicle according to claim 16 for the delivery of a therapeutic molecule to a target site.

22. A method of treating cancer, comprising administering to a subject in need thereof an effective amount of the delivery vehicle of claim 17 wherein the delivery vehicle comprises an anti-cancer drug, and triggering the release of said anti-cancer drug.

Fig. 1

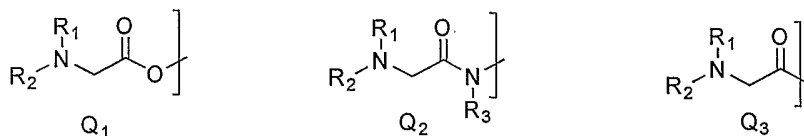
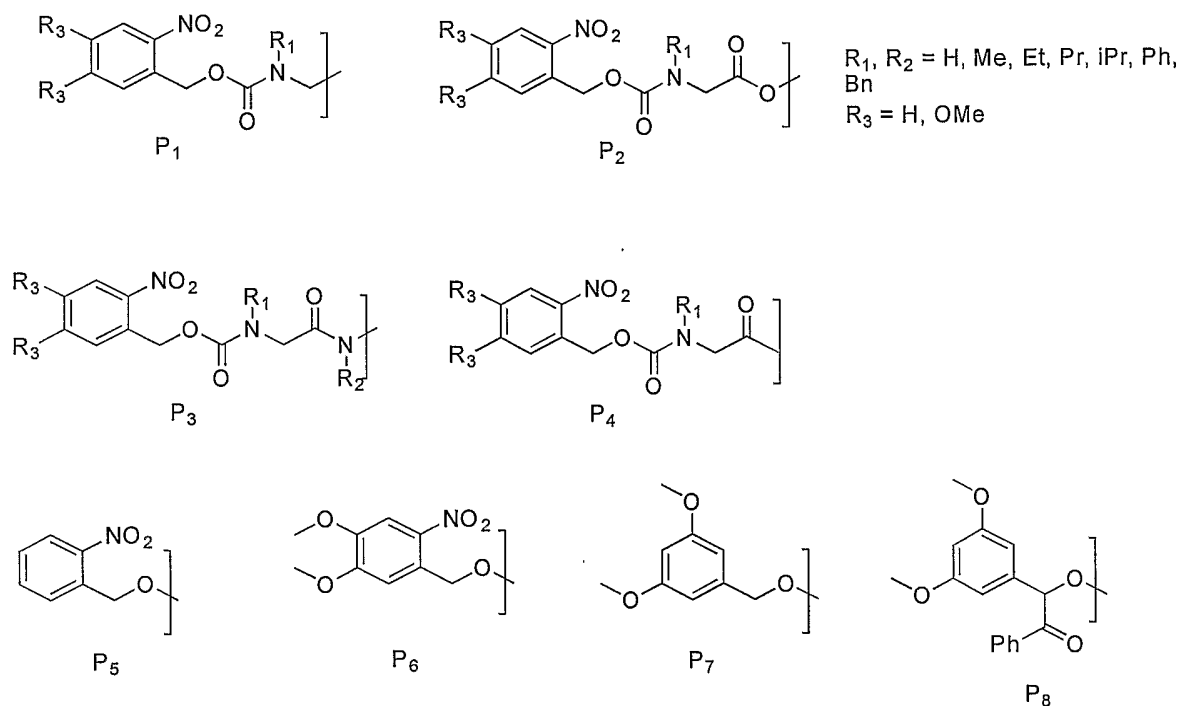
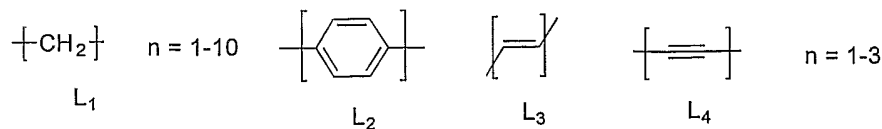
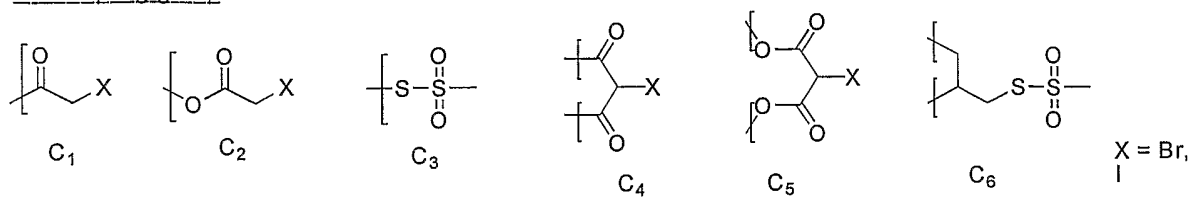
Q - chargeable groupP - photocleavable chargeable groupL - linker groupC - coupling group

Fig. 2

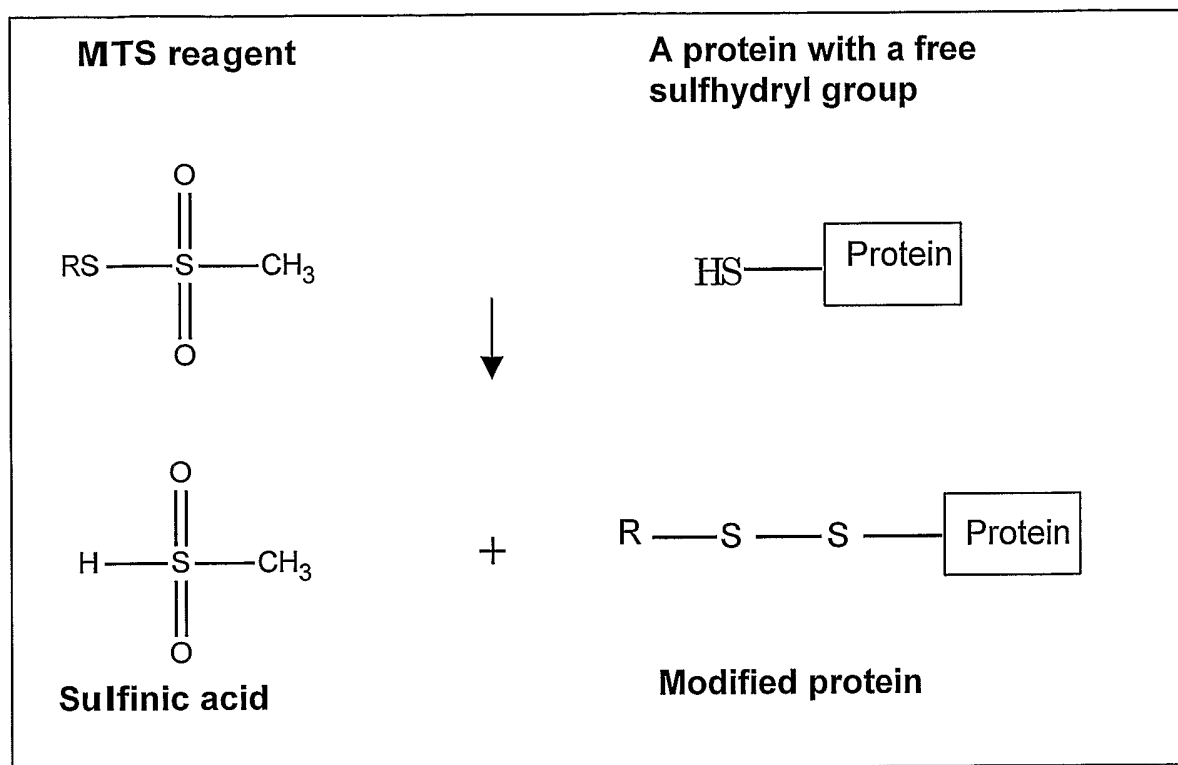


Fig. 3

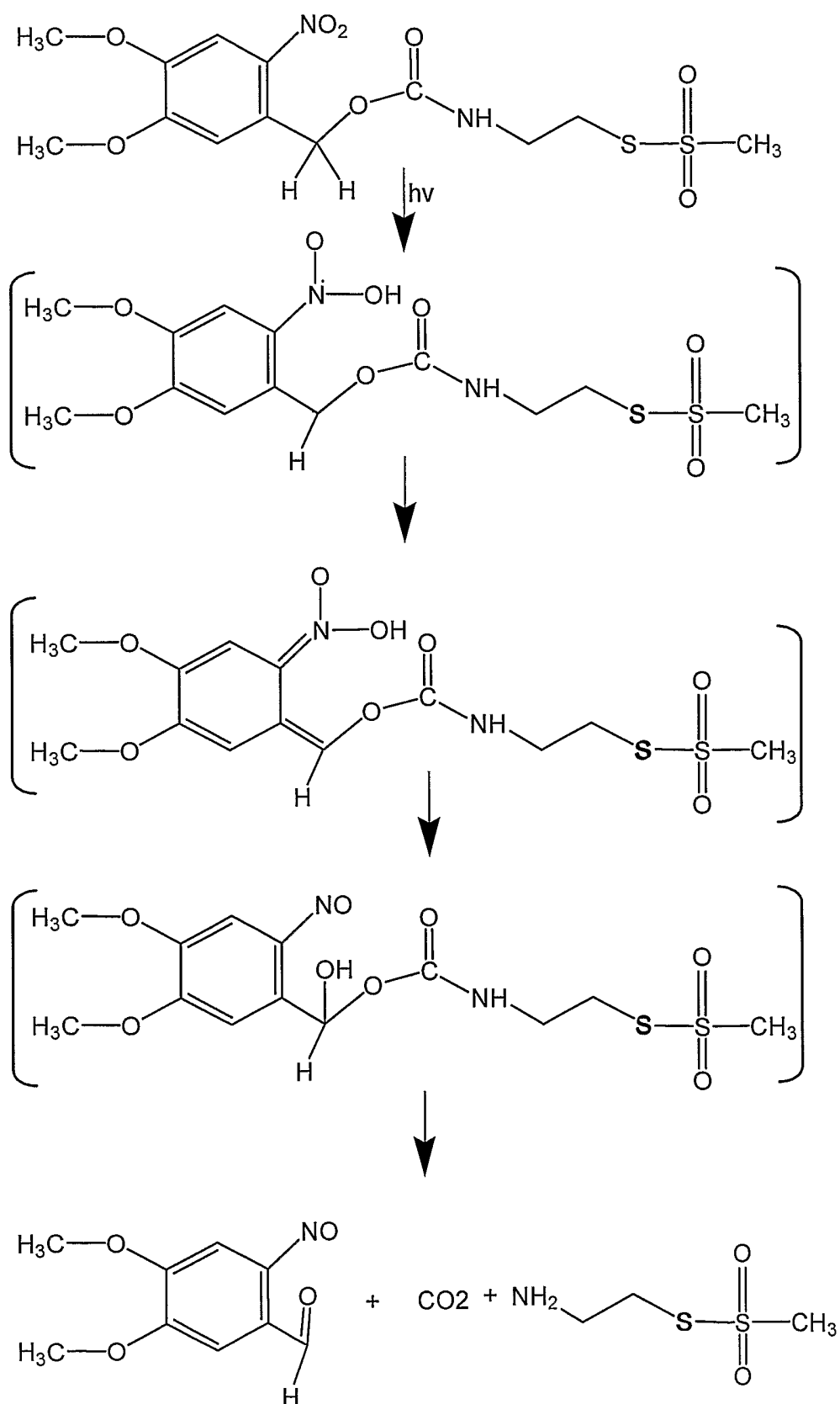


Fig. 4

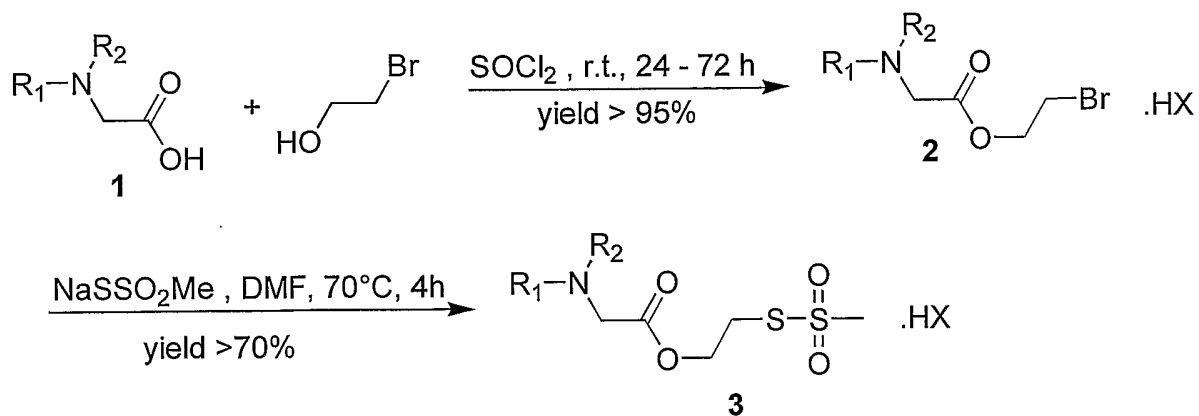
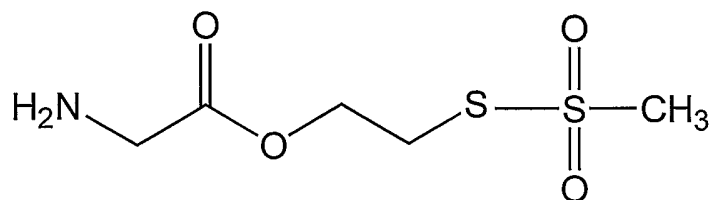
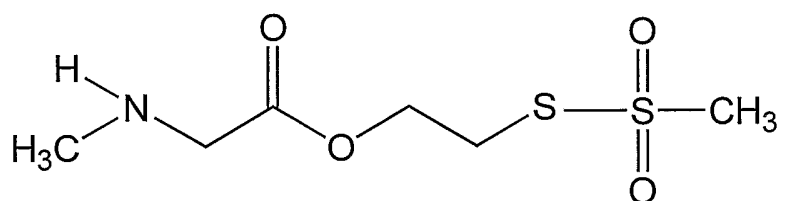


Fig. 5

A)



B)



C)

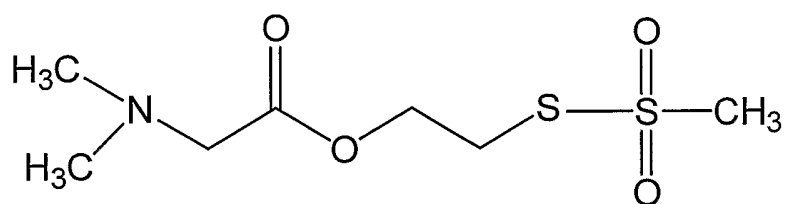


Fig. 6

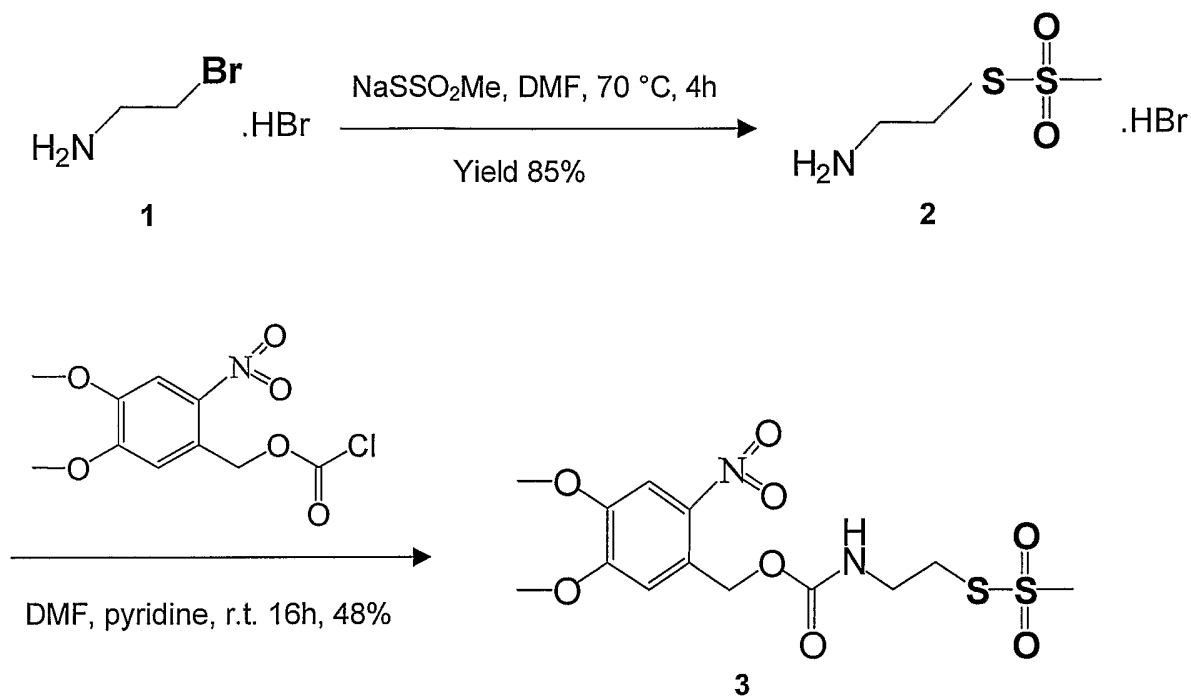
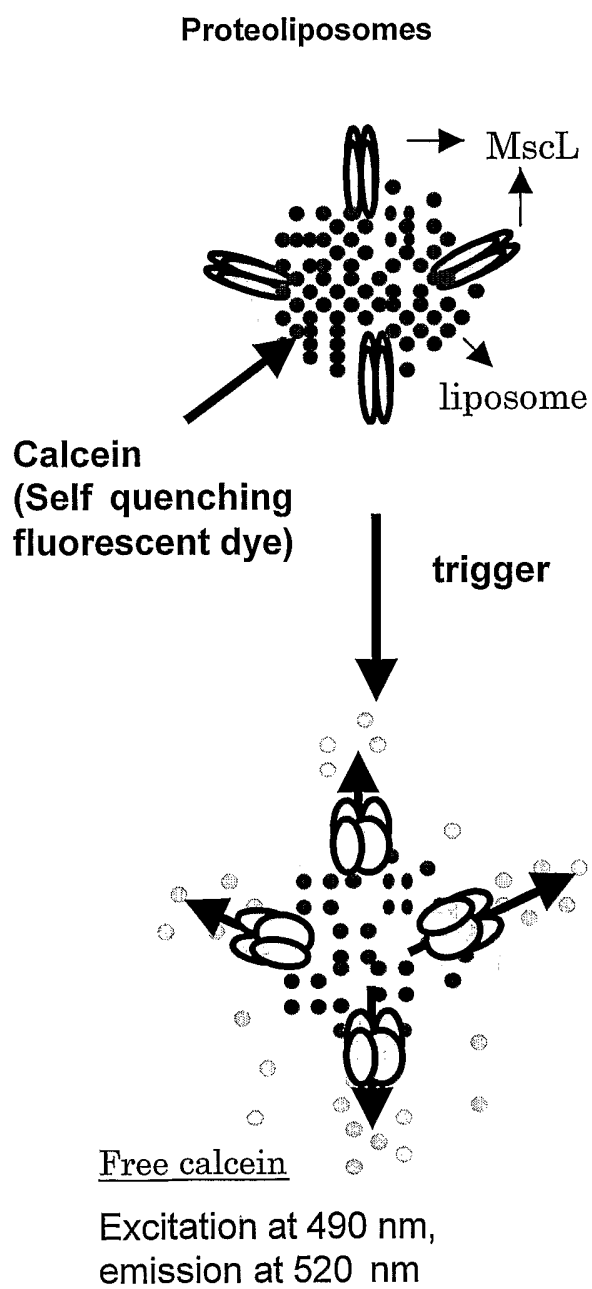
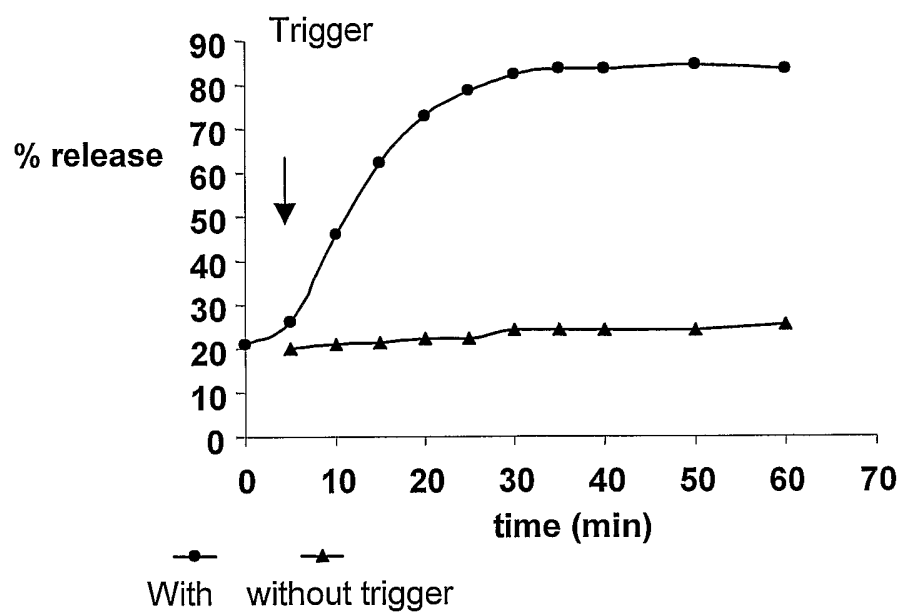


Fig.7B



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Fig. 7C



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Fig.8A

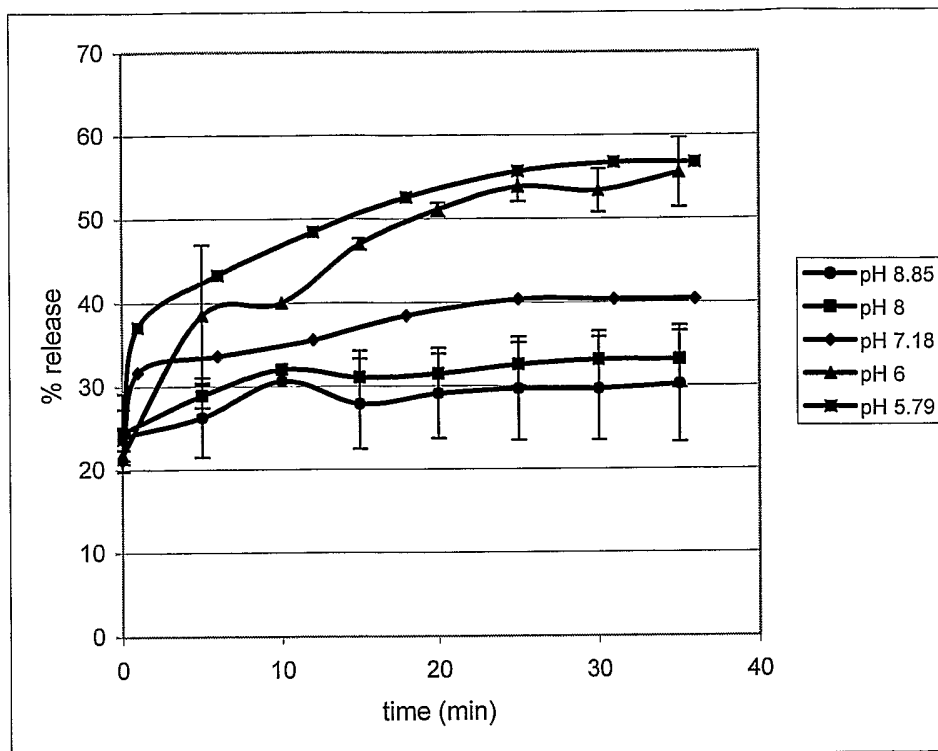
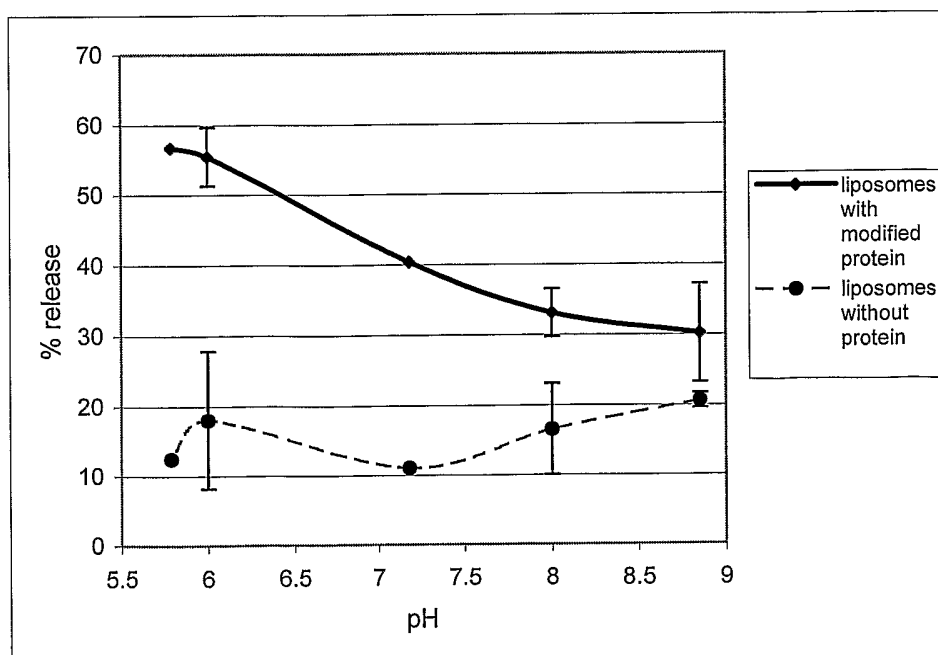


Fig. 8B



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Fig. 9A

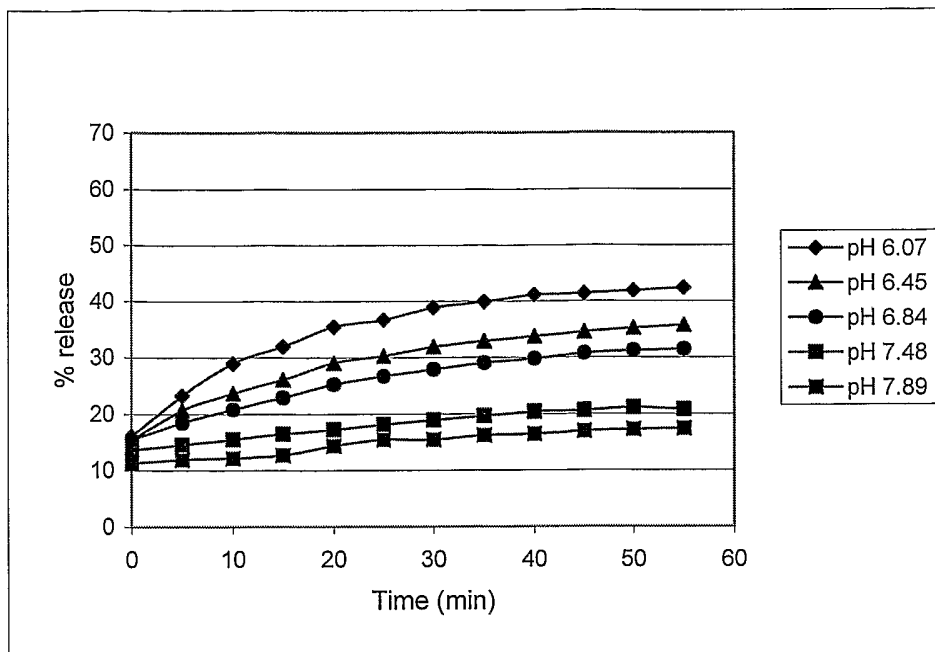
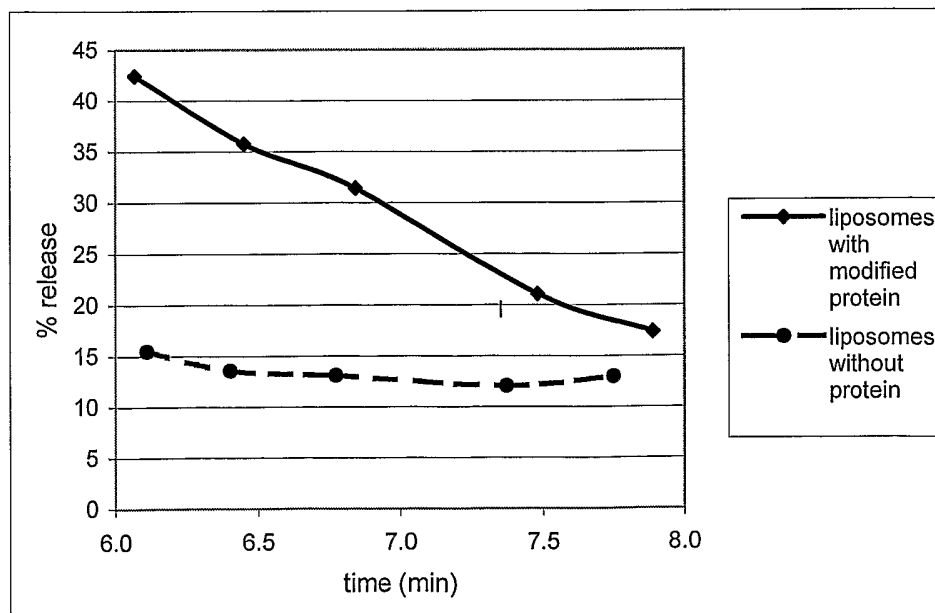


Fig. 9B



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Fig. 10A

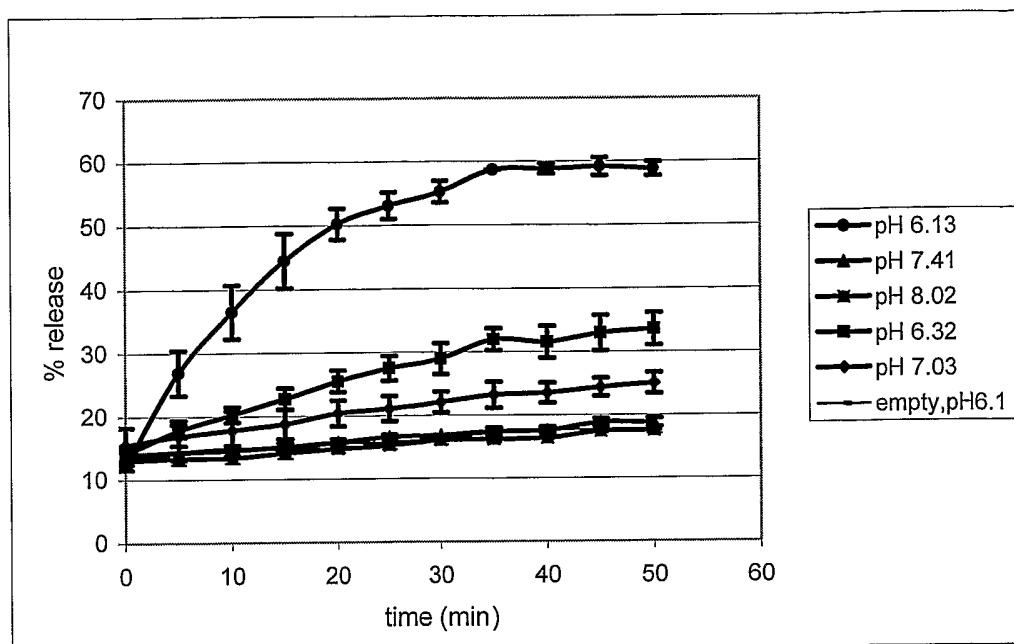


Fig. 10B

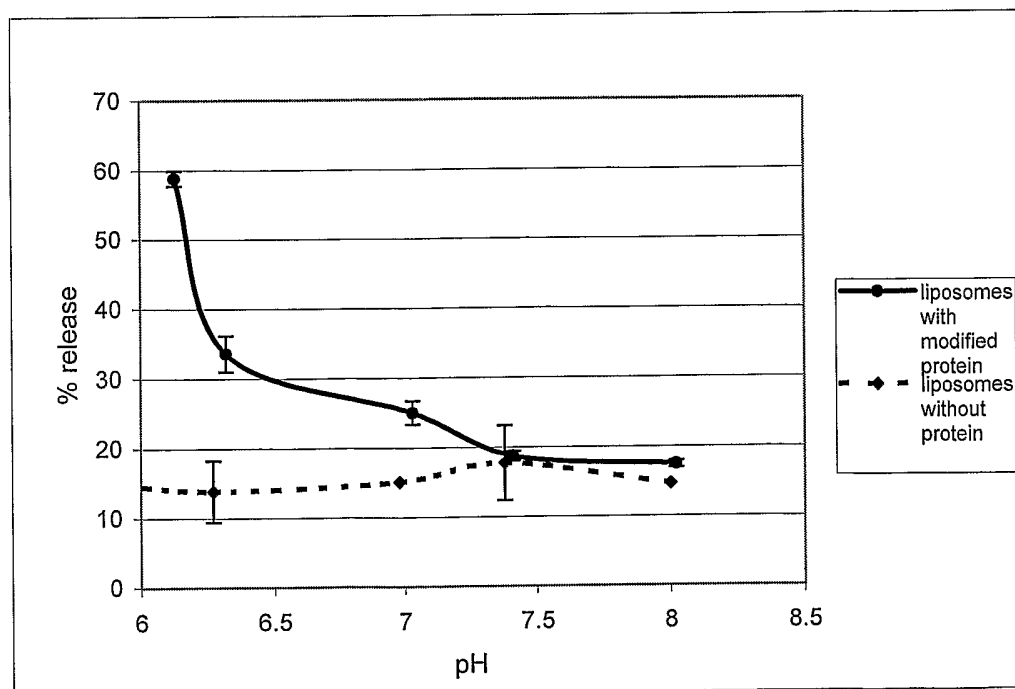


Fig. 11A

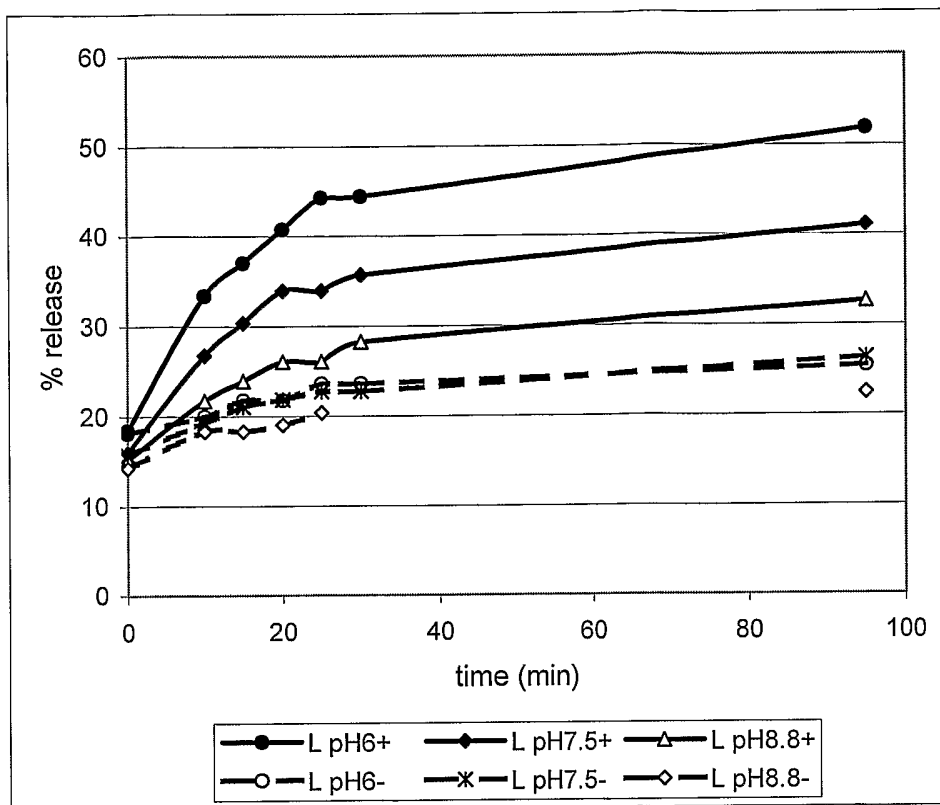


Fig. 11B

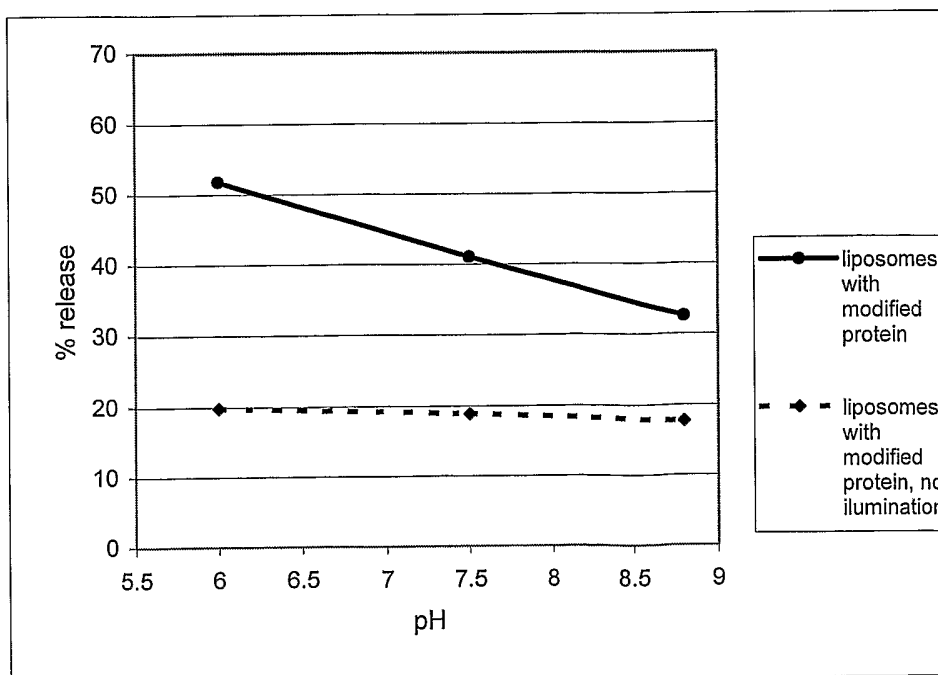


Fig. 12

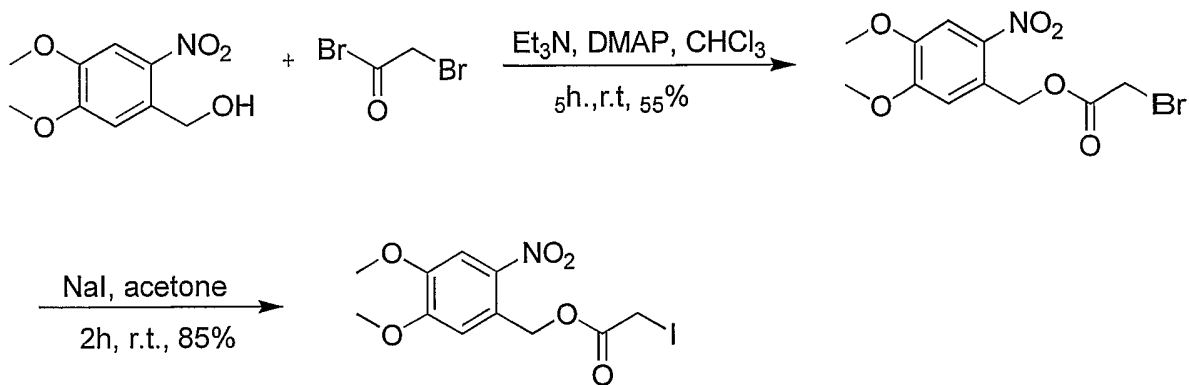


Fig. 13

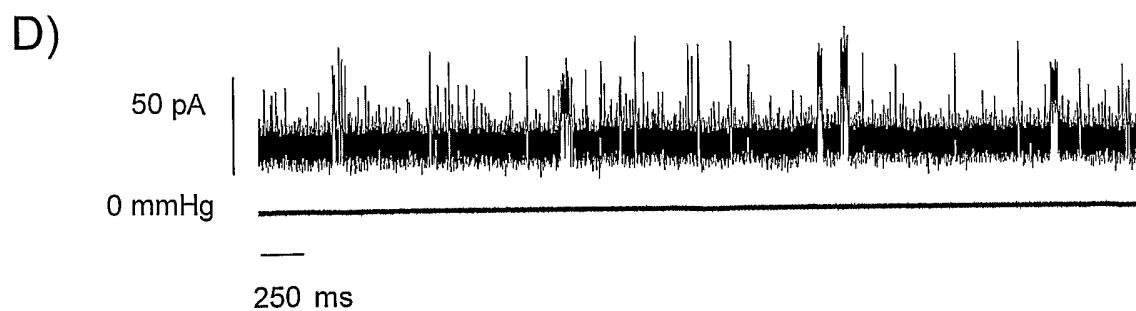
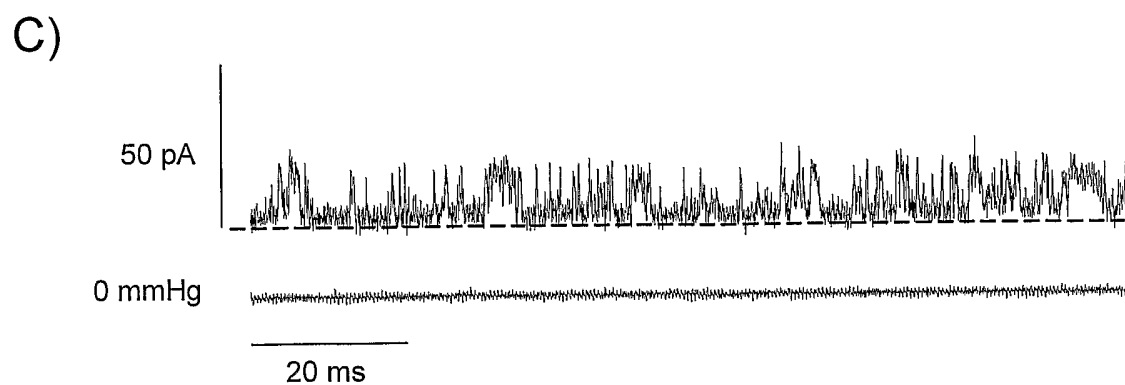
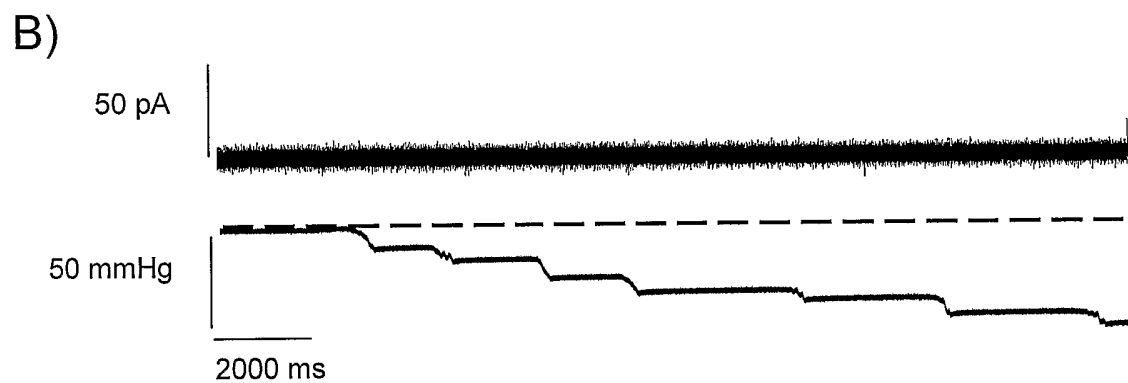
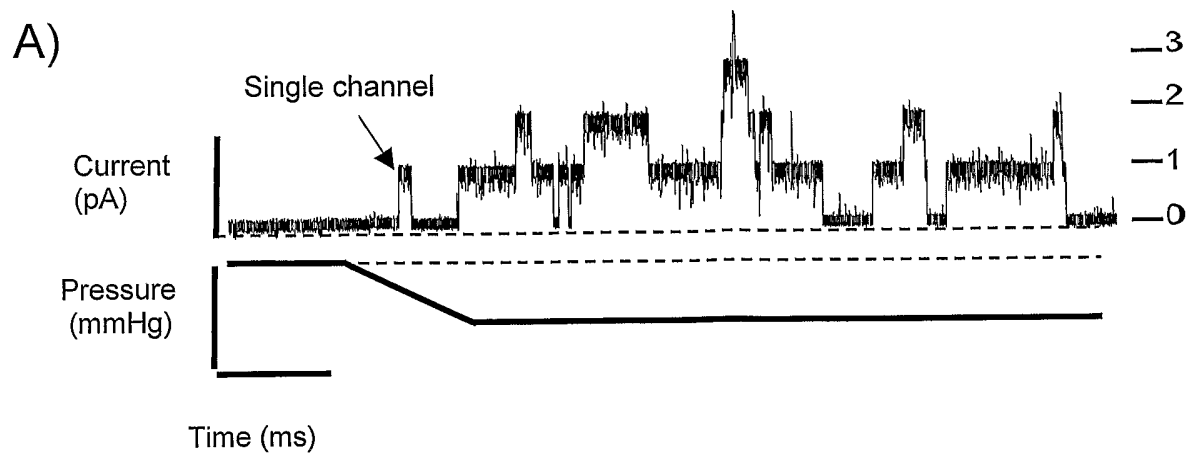
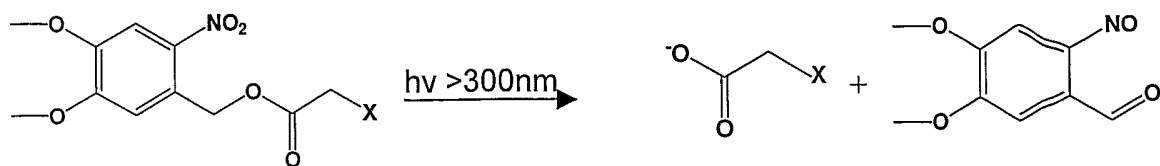
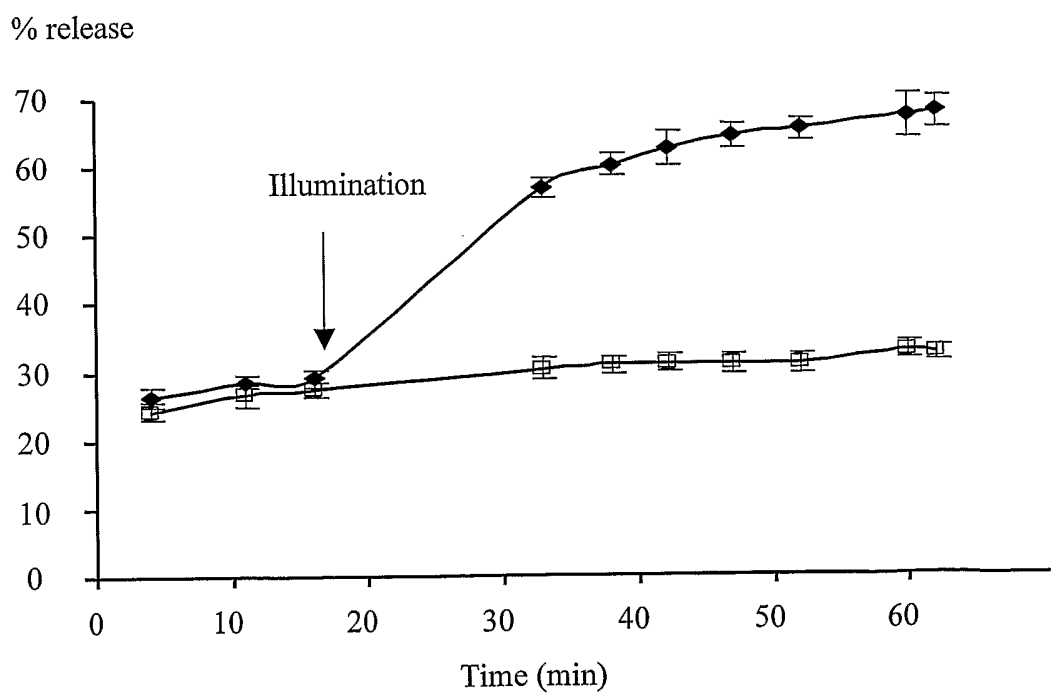


Fig. 14A



X = I or MscL channel protein

Fig. 14B



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Fig. 14C

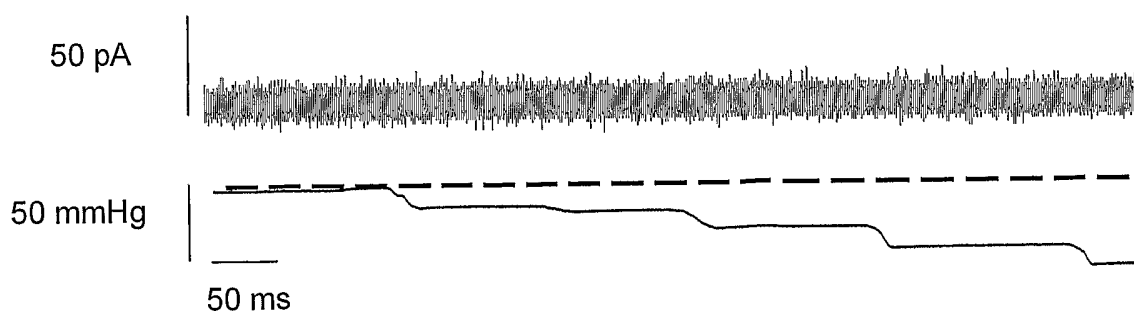


Fig. 14D

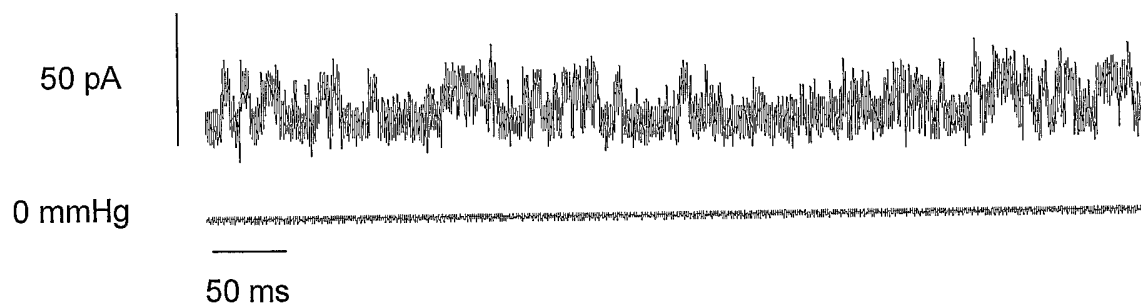


Fig. 15A

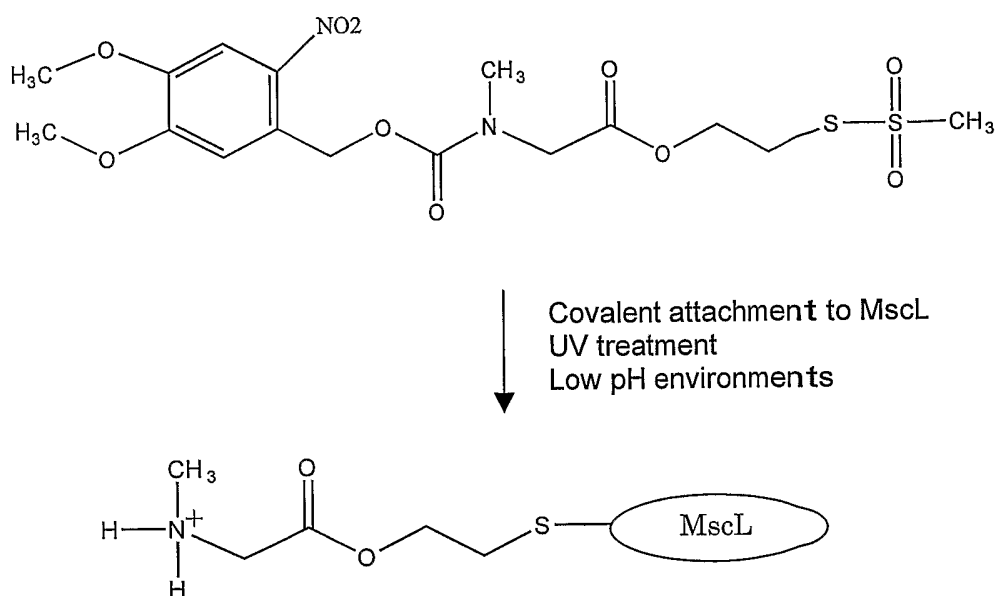
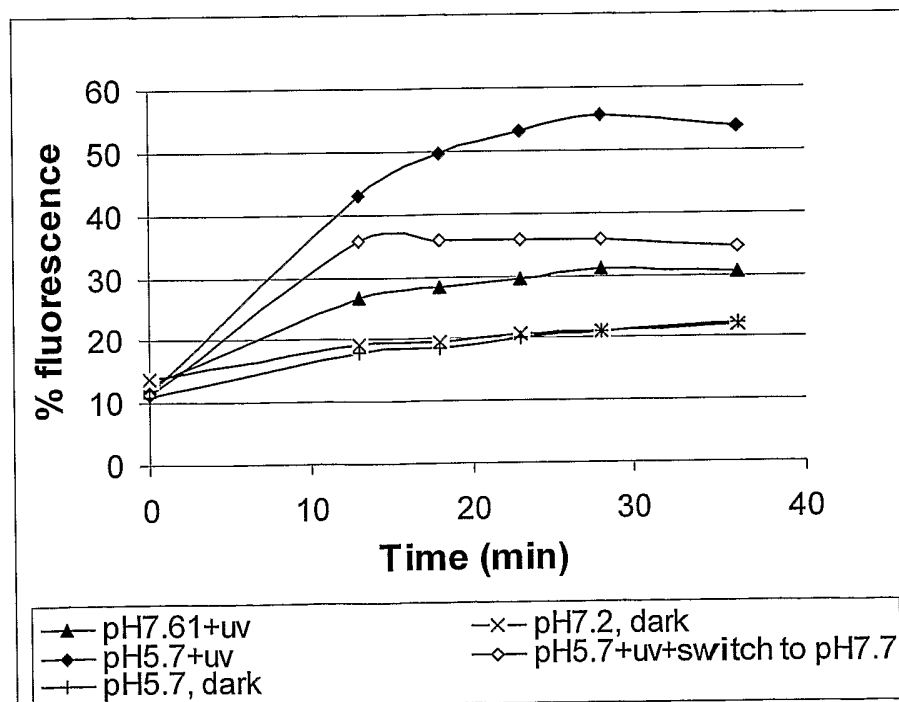


Fig. 15B



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Fig. 16A

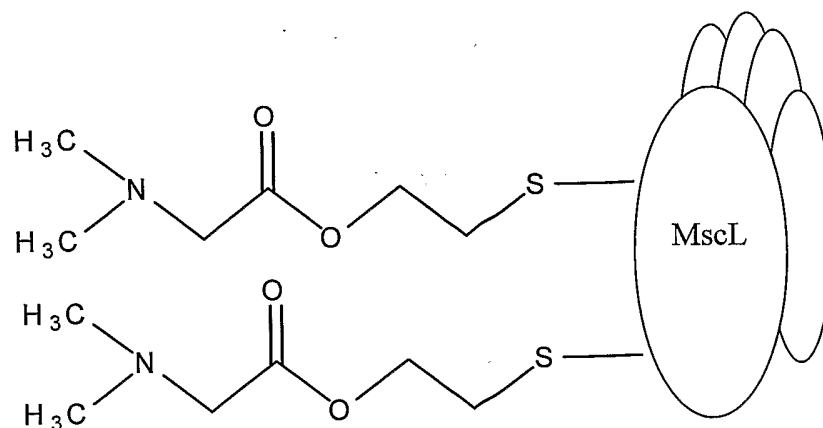


Fig. 16B

