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Rationally Designed Chemical Modulators Convert a Bacterial Channel Protein into a pH-Sensory Valve**

Armağan Koçer, Martin Walko, Erna Bulten, Erik Halza, Ben L. Feringa, and Wim Meijberg**

Small-molecule modulators are very promising tools for the exploration and manipulation of biological systems beyond the limits of genetics. The modern molecular biology toolkit provides a variety of methods that aid in exploring and understanding the structure and function of biological molecules. However, these methods have limitations, especially in the range of changes and responses that they can accomplish. In this regard, chemical modification provides a complementary approach. Variations can be introduced to the protein to confer features not achievable with the 20 amino acids that are genetically encoded. This is especially true when a combination of properties, such as reversibility, tunability, target specificity, sensitivity to external stimuli, and control over the timescale of the effect, are desired all at the same time.

A particularly interesting property to control at the molecular level is transport across barriers such as biological membranes, as such control could easily lead to applications in, for example, sensing and detection and drug delivery. This effect has been pursued in a number of studies on the construction of functional nanopores with either synthetic molecules or naturally occurring channels, the latter mainly as β-barrel structures.[1] Herein, we describe the rational design

[*] M. Walko,[+] E. Halza, Prof. Dr. B. L. Feringa
Department of Organic and Molecular Inorganic Chemistry
Stratingh Institute and Material Science Center
University of Groningen
Nijenborgh 4, 9747 AG, Groningen (The Netherlands)
Fax: (+31) 50-363-4296
E-mail: b.l.feringa@rug.nl

Dr. A. Koçer,[+] E. Bulten, Dr. W. Meijberg
BiOMaDe Technology Foundation
Nijenborgh 4, 9747 AG, Groningen (The Netherlands)
Fax: (+31) 50-363-4429
E-mail: meijberg@biomade.nl

[+] These authors contributed equally to this work.

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and engineering of a semisynthetic α-helical channel protein by altering its intrinsic properties, such that it senses changes in ambient pH and converts this signal into the opening of a pore (Figure 1). To do so, chemical modulators were developed that allow tuning of the pH interval and sensitivity of the response (that is, steepness of the transition). Furthermore, the introduction of a photocleavable protecting group results in a light-activated pH-sensitive valve.

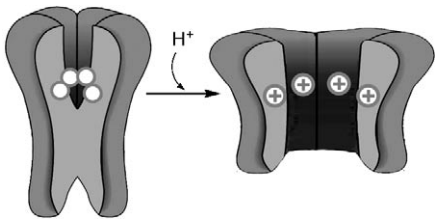


Figure 1. Protonation-induced opening of a mechanosensitive channel, which shows four of the five subunits in the pentamer (conceptual drawing). Protonation of a hydrophobic pore region of the MscL results in conformational changes and channel opening.

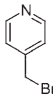
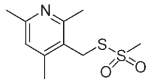
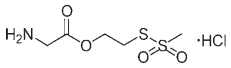
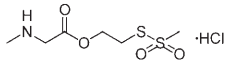
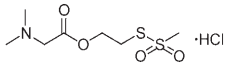
The protein we used is one of the best-studied channel proteins to date, that is, the mechanosensitive channel of large conductance (MscL) from *Escherichia coli*.[2] In nature, this homopentameric protein is embedded in the cytoplasmic membrane[3,4] and protects the bacterial cell against severe osmotic shocks.[5] Under hypo-osmotic conditions, cell turgor leads to tension in the membrane and an altered lateral-pressure profile, which in turn triggers large conformational changes.[6] The result is a 3-nm nonselective pore,[7] through which molecules can flow to balance the osmotic difference between the interior and exterior of the cell.[8]

An important parameter that influences the gating transitions of the MscL is the polarity of the hydrophobic constriction zone.[9] An increase in the polarity or hydrophilicity of the amino acid residue 22, located in this part of the protein, shifts the tension threshold for channel opening to lower values.[10] The introduction of a cysteine residue at this position followed by chemical modification with charged sulfhydryl-reactive compounds caused the channel to gate spontaneously.[11] Recently, we used covalently attached photosensitive modulators to induce charge at this position, thus leading to a channel in which gating could be switched on and off in the absence of any applied tension in response to radiation of an appropriate wavelength.[12]

Herein, we coupled charge-induced channel opening of the MscL to the ambient pH. In vivo this parameter varies depending on the health status of the surrounding tissue and/or cellular compartment. Around solid tumors, sites of inflammation, endosomes, and lysosomes the pH is lower (pH 6.8 to 5)[13] than under normal physiological conditions (pH 7.4). The incorporation of pH-controlled membrane channels in drug-carrying liposomes will allow selective release at diseased sites only. This might increase the effectiveness of the drug-delivery device, which is our ultimate goal. In previous studies it has been shown that a glycine-22-to-histidine (G22H) mutant exhibited increased sensitivity toward membrane tension but did not open spontaneously in response to pH.[10] To achieve pH-induced opening we modified the channel with specifically designed pH-responsive chemical modulators. The pH-sensitivity interval of the channel could be tuned by varying the pK_a and hydrophobicity of the modulators. Further control over the timing, location, and amplitude of the channel opening was obtained through the use of caged modulators, in which the pH modulator was protected by a photoremovable group. Channels thus modified are inert to the environmental pH until exposure to long-wavelength UV irradiation, at which point the pH responsiveness is immediately regained. This additional feature resulted in more flexibility and precision with respect to external control over the channel opening and the associated release of molecules from carrier devices.

To selectively attach the chemical modulators to the protein, glycine-22 was mutated into cysteine (G22C).[11] This change resulted in five identical modification sites within the hydrophobic constriction zone of the homopentameric protein. The chemical modulators were designed as sulfhydryl-reactive molecules (Table 1) and were covalently attached to detergent-solubilized MscL-G22C. It is known that the constriction zone of the protein is not fully accessible to chemical modification when the MscL is in its natural membrane environment.[20] ESI-MS analyses (see Supporting Information) indicate that in detergent-solubilized systems under our conditions all five subunits of the protein are modified simultaneously, and that the modification proceeds specifically and quantitatively within the detection limits of the mass spectrometry measurements. Our results are indicative of a more relaxed conformation of the channel in detergent solution compared to the natural membrane environment. In this regard, it is interesting to recall the arguments about whether the *Mycobacterium tuberculosis* crystal structure,[21] which was obtained in the presence of detergent, represents a fully or partially closed conformation

Table 1: The structure and properties of synthetic pH modulators.

	Compound	pK_a	Mass[a]	
			calcd	exptl
1		5.23[14]	15 786	15 789
2		7.10[15]	15 860	15 860
3		7.75[16]	15 828	15 830
4		7.85[b]	15 842	15 841
5		7.35[b]	15 856	15 855

[a] The masses refer to the monomer of MscL-G22C covalently modified with the corresponding compound. [b] The pK_a values were estimated on the basis of similar molecules found in the literature.[17–19].

of the protein.[6,22,23] Our results support the latter interpretation.

The effects of chemical modification of MscL have been followed under isoosmotic conditions in a straightforward liposomal efflux assay, by using fluorescence changes upon the release of a reporter (see Supporting Information). Briefly, chemically modified channels were incorporated in liposomes consisting of synthetic lipids by a detergent-mediated reconstitution method[24] in the presence of calcein, a self-quenching fluorescent dye. After chromatographic removal of external dye, the resulting proteoliposomes (average size 200 nm, as determined by dynamic light scattering) were analyzed for channel activity at different pH values. Channel opening led to the release of the dye from the liposomal interior, and the resulting concentration decrease and dequenching upon entering the surrounding buffer could be monitored as an increase in fluorescence. Controls with liposomes lacking MscL-G22C, or containing unmodified MscL-G22C or wild-type MscL (with the C-terminal His tag that is present in all proteins described here), did not show any release activity (see Supporting Information).

pH-induced release through MscL modified with **1** could not be observed by the assay described above between pH 6 and 8, the pH limits of the assay. To access lower pH ranges, pyridine-modified channels were analyzed at the single-molecule level by measuring the ionic current flowing through the channel in response to the pH in patch-clamp experiments. Spontaneous channel opening was observed at pH 5.2, but at higher pH values the channels still required tension to open (see Supporting Information). Modulator **2** was designed to have a higher pK_a value, within the physiological range as well as the pH interval of the efflux assay. Channels modified with **2** did indeed show pH-dependent activity in efflux assays (Figure 2a), a result confirmed with patch-clamp measurements (Figure 2b and c). Thus, we showed for the first time that it is possible to open the channel in response to pH in synthetic lipids without applying any tension, in both patch-clamp and liposomal efflux setups.

It is known that the gating behavior of MscL channels can be correlated with the hydrophilicity of the residues present in the pore constriction.[10] From this point of view, the pyridine derivatives are not ideal modulators as they are quite hydrophobic. To address this issue and improve the liposomal efflux efficiency, pH modulators based on a different structural motif (**3**–**5**) were designed. We aimed specifically at increased hydrophilicity, a pK_a value tunable in the range pH 6–8, synthetic availability, and specific and efficient coupling to the protein.

Proteoliposomes containing channels modified with modulators **3**–**5** responded to pH and released more calcein at pH values below the pK_a of the modulator used. In contrast, control liposomes without MscL were stable at all pH values analyzed (Figure 3). The pH interval in which the channel is activated is dependent on the pK_a and hydrophobicity of the modulator. When the hydrophobicity of the pH modulator was higher as a result of the additional methyl groups, the modified channel was harder to open at higher pH values, where the modulator is mainly in its uncharged form. A particularly interesting case is the channel modified with **5** (Figure 3c), which has the highest hydrophobicity of the three modulators as well as the lowest pK_a. At pH 7.4, the

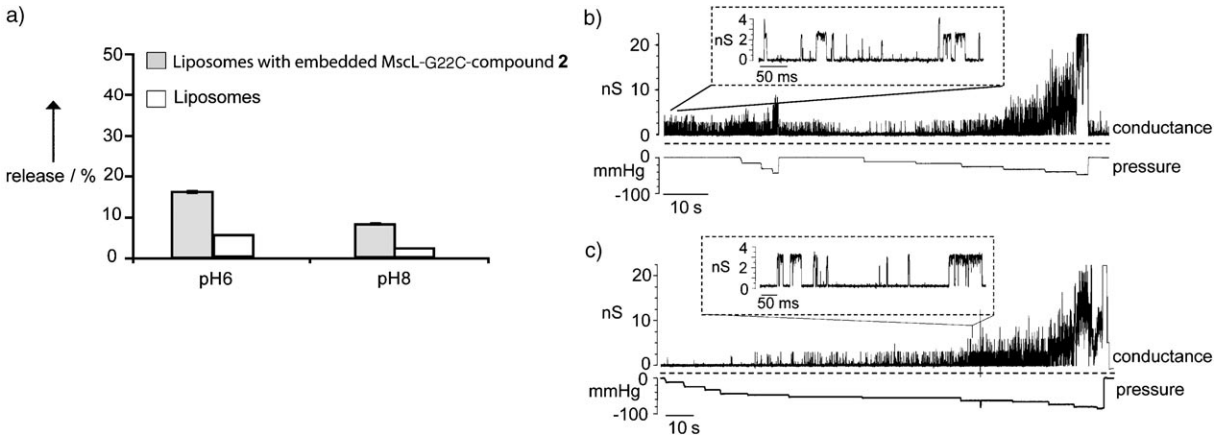


Figure 2. Activity of MscL-G22C modified with **2**. Modified MscL was reconstituted in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes at a protein/lipid ratio of 1:120 (wt/wt) with a detergent-mediated reconstitution method. The fluorescent dye calcein (final concentration 50 mM) was added to the lipid–protein mixture and the detergent was removed with biobeads. External calcein was removed by column chromatography on Sephadex G50 and the liposomal fraction was used in the efflux assay. Depending on the concentration, about 10 μL of liposomes were diluted in 2 mL isoosmotic buffer (330 mosm) with varying pH values, and the increase in fluorescence was monitored for 45 min. Maximum release was reached within 30 min and is reported here relative to the release obtained after bursting the liposomes with excess Triton X100. Error bars indicate the standard deviation of two separate experiments. a) pH-induced release in calcein efflux assays. At pH 8, the proteoliposomes released about 8% of the dye, but at pH 6 the release increased to 16%. Patch-clamp measurements at b) pH 5.8 and c) at pH 8. Insets: typical channel openings in an enlarged form. The channel opened spontaneously without applied tension at pH 5.8. The observed conductance was about 2.5 nS, but if tension was applied gradually, the channel also gated to its full conductance of 3 nS with as little as 15 mm Hg applied negative pressure. Conversely, at pH 8 spontaneous gating was absent and only the tension-dependent openings were observed. At lower tensions (up to −50 mm Hg pressure in the patch) the channel opened to lower subconducting states (1 and 2 nS), whereas at higher levels of applied tension the full conductance was reached. Further application of tension resulted in the opening of more than one channel.

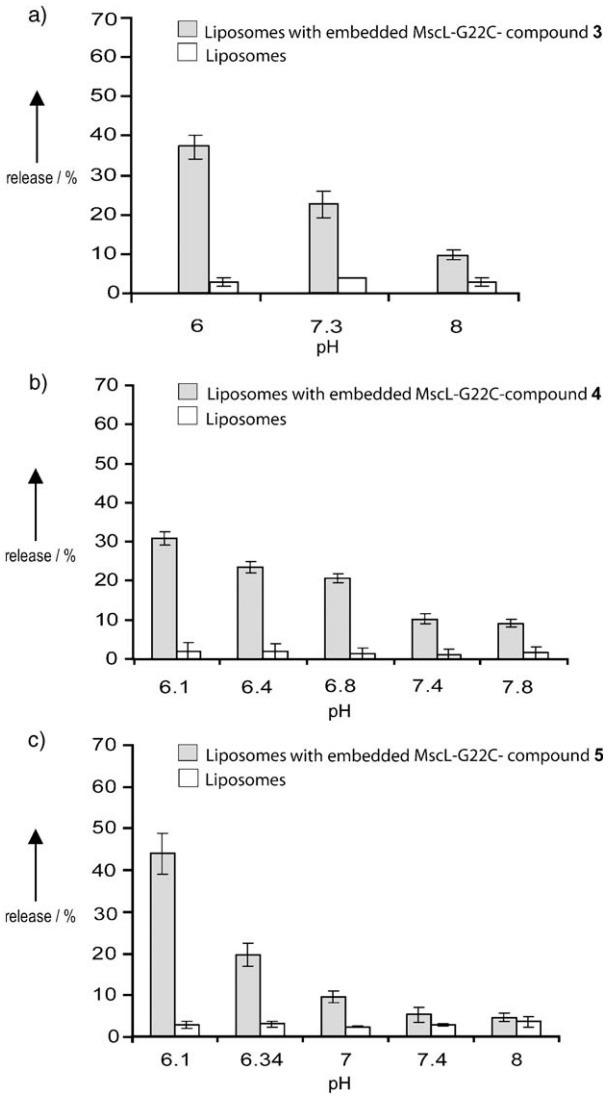


Figure 3. pH-dependent activity of modified MscL in calcein efflux assays. Modified MscL was reconstituted in DOPC/cholesterol/DSPE-PEG2000 (70:20:10 M) liposomes in the presence of calcein. The activity of the channel at different pH values was monitored to determine the maximum release values. Release was invariably completed within 30 min. Error bars indicate the standard deviation from four independent experiments. MscL-G22C modified with a) **3**, b) **4**, and c) **5**. DSPE = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; PEG = polyethylene glycol.

physiological pH for humans, liposomes with these channels retain their content at levels comparable to those of liposomes without protein, but start to open and release content at lower pH values, such as those found at cancerous and inflammation sites. The results were confirmed by patch-clamp measurements (see Supporting Information). As in the case of modification with **2**, the channel opened to a subconducting state in response to pH in the absence of tension, and to its full conducting state after the application of a small amount of tension. It has been reported that transition from the closed to the subconducting state represents the opening of the main gate of the channel and is associated with the highest energy barrier.[9] Our results, in particular the dramatic increase in channel activity upon a small amount of suction, appear to be in agreement with this model.

Further control over the pH-induced opening process was gained by chemically protecting the pH-sensitive group of the modulator. This allows activation on command. It was achieved by coupling the amino group of modulator **4** to a photolysable group that can be removed by irradiation with long-wavelength UV light (**6**; Figure 4a). In the dark, the

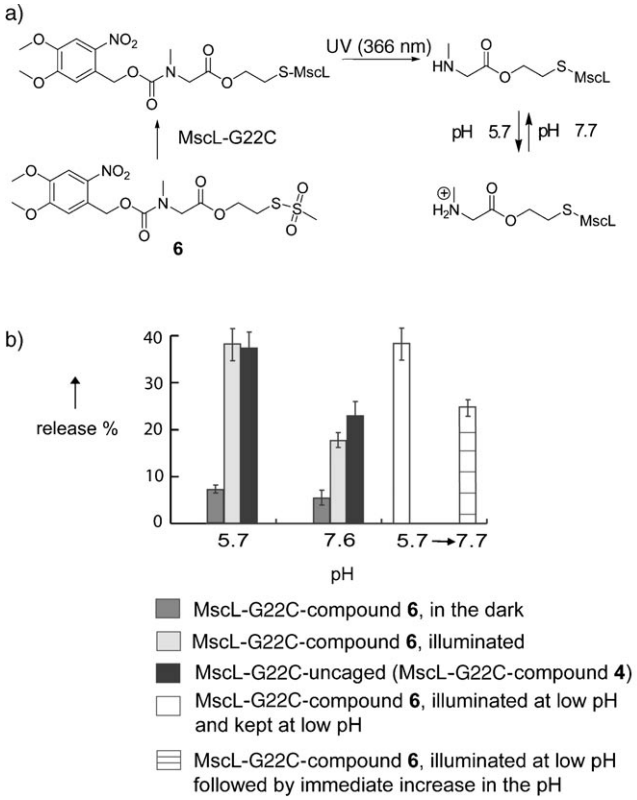


Figure 4. MscL-G22C modified with a caged pH modulator. a) Structure of **6** and removal of the protecting group by illumination. b) Activity of the modified MscL-G22C channels in the efflux assay. Channels modified with caged modulator **6** did not open at any pH value in the dark (dark gray bars). After deprotection by irradiation at 366 nm for 10 min (spectroline long-life filter, 365 mW cm^{-2}), channels became activated and released the liposomal content in dependence on the ambient pH (light gray bars). If the pH of the protein sample, which was illuminated at low pH, was immediately raised to 7.7 ± 0.1, the release of dye stopped (white bar with horizontal lines) because of closing of the channel; if the pH was kept constant at a low value, the channel stayed open (white bar) and release continued. Channels modified with uncaged modulator **4** opened in response to pH (black bar). Error bars indicate the standard deviation from three independent experiments.

modified channel remains closed and consequently calcein efflux experiments show no release of dye, irrespective of the environmental pH (Figure 4b, dark gray bars). After removal of the protecting group at the amine moiety by UV irradiation, the channel becomes responsive again to the pH of the environment, which results in high release of the liposomal content at low pH (Figure 4b, light gray bars).

Comparison of the performance of the illuminated sample with an uncaged sample at the same pH (Figure 4b, black bars) indicated that the expected activity of **4** after removal of its protecting group was recovered, and therefore that photocleavage was complete.

The caged modulator was also used to address the reversibility of the pH-induced opening of the channel. To this end, duplicate samples were illuminated at pH 5.7. Subsequently, the pH of one of the samples was raised to 7.7. Although release started during illumination in both samples, the process was arrested immediately after the pH was raised to 7.7 (Figure 4b, white bar with horizontal lines), while continuing at low pH (Figure 4b, white bar). This experiment shows that the modified pH-sensitive channels can be closed through a protonation reaction. Unfortunately, the time course and sensitivity of the liposomal release experiment do not allow this cycle to be repeated a number of times with the same sample. Therefore, further investigation is required to conclusively prove that the pH-induced opening of the channel is reversible, that is, that the closed state at high pH after removal of the cage is identical to the closed state after a cycle of low and high pH.

The pH modulators described herein were able to confer pH responsiveness to the channel, as shown from patch-clamp and efflux measurements. However, the measurements also show that complete release of enclosed calcein was not achieved. Although this effect is not fully understood, a number of explanations can be put forward. For example, the reconstitution procedure does not guarantee that an active channel is associated with every liposome. Furthermore, in the case of the pH modulators, the ionization of one group will depress the pK_a of its neighbors in close proximity in the homopentameric protein. The accumulation of five positive charges will therefore only be achieved at much lower pH values, and hence the overall activity of the protein will appear to be lower. This proposal was confirmed by showing that modification of the protein with [2-(trimethylammonio)ethyl]methanethiosulfonate ($MTSET^+$), which carries a charge under all experimental conditions used here and thus results in five positive charges in the channel constriction, leads to maximum release values close to 100% (see Supporting Information). Finally, it has been reported that when the number of MscLs was low in the patches, the channel activity was followed by irreversible inactivation.[25] A combination of the latter two effects, that is, reduced activity and inactivation with time, will lead to release values below 100%.

In summary, we have converted a bacterial channel protein into a pH-sensitive valve. When embedded in liposomes, the modified channels sense the ambient pH and conditionally release the liposomal content. The sensitivity and pH interval for channel opening were tuned by varying the hydrophobicity and pK_a of the modulators. Additional control over pH response was gained through the introduction of a photosensitive caging group. Gaining control over MscL channel activity as presented herein is valuable for further investigations into the mechanism of the channel function. The pH-activated channel or caged channels can also serve as control valves to release or mix the contents at a desired location, time, and dosage, for example, in micro/nanosensory and delivery devices.

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