pH-Responsive Release

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pH-Responsive Release of Acetal-Linked Melittin from SBA-15 Mesoporous Silica**

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Mesoporous silica materials have recently attracted much interest as potential carriers for drug delivery.[1–3] Their low toxicity combined with their high surface area and large accessible pore volumes make them suitable as transporters for anticancer drugs or target-specific agents. An important feature of such systems is the controlled release of compounds from the host material only at the desired location and at a specific time. For this purpose, the porous host should respond to specific external triggers, such as changes in the pH value upon entry into the cell, changes in temperature, or a change in the redox potential of the environment.

In recent years, several research groups have succeeded in the development of trigger-responsive valves.[4–9] In most cases, large molecules or nanoparticles were attached reversibly to the pore mouths to sterically hinder loaded guest molecules (drugs or model compounds) from leaving the pores. For example, acetal-gated mesopores were used in one study as pH-responsive gatekeepers for mesoporous silica.[8] Another approach has demonstrated that coumarin derivatives can act as photoresponsive gatekeeper molecules for MCM-41. Irradiation of the coumarin-functionalized silica surface with UV light ($\lambda > 310$ nm) caused a [2+2] photocycloaddition between two coumarin molecules, thereby leading to an efficient closing of the pores. Reopening was achieved by irradiation at higher energy ($\lambda \approx 250$ nm).[9]

Another approach to create delivery-on-demand systems based on mesoporous materials is to directly attach the therapeutic molecules to the silica pore walls by using reversible reactions. In one recent approach, disulfide-bridged linkers were used between the pore wall and cysteine residues in mesoporous silica nanoparticles to generate a redox-responsive delivery mechanism.[2] We have contributed to this field by studying the cell uptake and drug-release mechanism of this method in detail.[10] An additional highly desirable option for biomedical applications would be to have pH-responsive linkers between the substance to be delivered and the porous host, since the pH value drops from 7.4 to 5.5 upon uptake into the endosome.[11]

Here we describe the use of acetal-linkers for a study of the pH-responsive release of melittin from SBA-15-type mesoporous silica. Melittin, the active component of bee venom, is a small peptide containing 26 amino acids. This compound is currently being widely investigated in the field of nanomedicine because of its lytic or apoptosis-inducing properties.[12,13] It acts as a membrane-invading agent, which leads to a local disruption of lipid membranes. It has previously been used as an endosomolytic peptide for siRNA–polymer conjugates (siRNA = small interfering RNA).[14] The radius of gyration of this small peptide of 1.3 nm makes it suitable for encapsulation in mesoporous host materials.[15]

The structure of certain acetals offers a reasonable stability under extracelullar conditions while undergoing fast hydrolysis in an acidic milieu. The applicability of acetals to controlled-release systems was recently demonstrated with oligoethyleneimine conjugates.[16] It was shown that acetal-linked oligoethylene imines can be used as efficient pH-sensitive gene carriers. Another research group has applied this approach to create pH-sensitive polyethyleneoxide conjugates of model drugs.[17] Acetal-linked proteins have not yet been reported for protein delivery using mesoporous structures.

As the host material, we used large-pore SBA-15-type mesoporous silica. The material was produced according to published procedures.[18] A mixture of Pluronic 123, cetyltrimethylammonium bromide (CTAB), mesitylene, potassium chloride, and hydrochloric acid was used to hydrolyze tetraethylorthosilicate (TEOS) and assemble the mesoporous structure. After calcination, the nitrogen sorption isotherm gave pore diameters of about 11.1 nm (according to nonlocal density functional theory) and a Brunauer–Emmett–Teller (BET) surface area of about 700 $m^2 g^{-1}$. The resulting material was subsequently functionalized with 3-mercaptopropyltrimethoxysilane (MPTS) by post-synthetic grafting in toluene at reflux under nitrogen to yield sample SBA-SH (detailed experimental procedures can be found in the Supporting Information). Thermogravimetric analysis (TGA) was performed to determine the amount of functional groups grafted to the mesoporous host. A mass loss of 5% during heating to 900 °C was evaluated to correspond to a thiol content of 0.67 mmol of mercaptopropyl moieties per gram of material (the respective isotherm and TGA curves can be found in the Supporting Information).

The presence of thiols was also confirmed by cleavage of 2,2′-dithiopyridine, which released the yellow 2-thiopyridon, as described in the literature.[2] The SBA-SH was dried under

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vacuum and was then suspended in water-free dimethylsulfoxide (DMSO) in a nitrogen atmosphere. The sample was first divided into two aliquots of five milligrams each and the samples were incubated with a 10-fold excess of either the MK or BM linker relative to the thiol content (samples SBA-MK and SBA-BM, respectively; Figure 1). The reactions were carried out for 14 h under nitrogen at room temperature, followed by washing with DMSO. The increasing functionalization of the material was monitored by IR spectroscopy (Figure 2). The presence of the maleimide-linked MK moiety can be observed by the emerging C=O stretching band at 1707 cm^{-1} (Figure 2b). In contrast, the sample SBA-BM also shows aliphatic ether vibrations around 1450 cm^{-1} (Figure 1c). The C=C stretch of the maleimide is also visible in the SBA-MK sample. The signals below 1250 cm^{-1} can be attributed to vibrations of the silica framework (the IR spectra of the pure linkers used as a reference can be found in the Supporting Information).

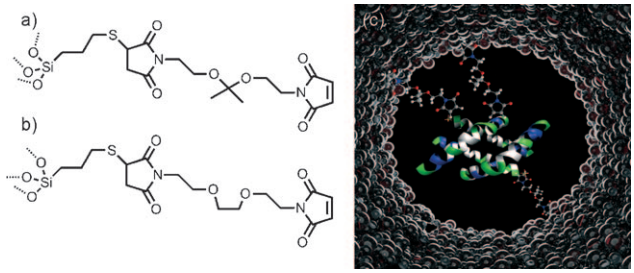

Figure 1. Linkers in a) SBA-MK and b) SBA-BM. c) A three-dimensional representation of the pH-sensitive attachment of melittin to the pores of SBA-15.

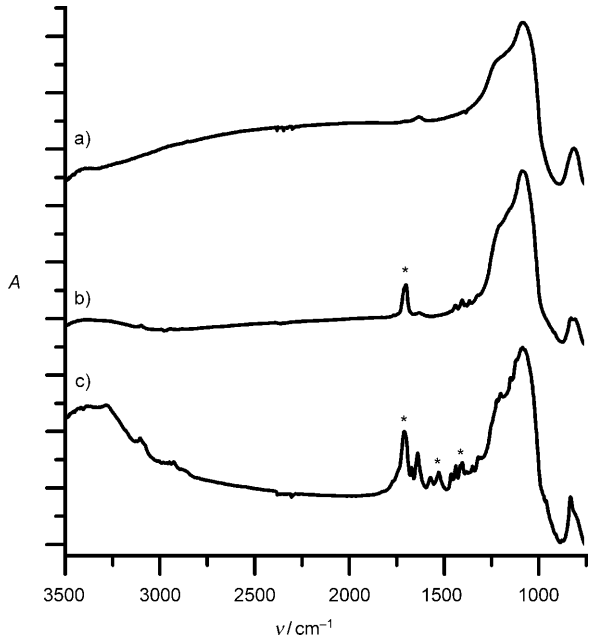

Figure 2. IR spectra (offset, normalized to the Si–O stretch) of the samples a) SBA-SH, b) SBA-MK, and c) SBA-BM. Bands discussed in the text are marked with asterisks.

A negative Ellman's assay also implies that the free thiol groups had reacted with the maleimide groups of the respective linkers. Subsequently, 1.0 mg of each sample was incubated at room temperature for 4 h with a 1.5-fold excess of synthetically produced, cysteine-terminated melittin (samples SBA-MK-Mel and SBA-BM-Mel). The conjugation reaction was carried out under water-free conditions in DMSO to avoid unwanted cleavage of the acetal linker. The samples were analyzed after intensive washing and centrifugation.

A photometric approach was used to quantify the amount of incorporated or released melittin.[19] This approach is based on the prediction of the molar extinction coefficient ε(280) of a peptide by taking into account the number of tryptophan and tyrosine residues in the primary structure of the peptide and the number of disulfide bridges present. According to Ref. [19], every tryptophan residue contributes 5500 $M^{-1}cm^{-1}$ to the value of ε. Since the primary structure of melittin contains only one tryptophan residue and none of the other relevant groups, we can set $\varepsilon(280)$ to 5500 $M^{-1}cm^{-1}$. For the experiment, 1 mg of SBA-MK was incubated with 3 mg of melittin in 1.5 mL of water-free DMSO for 4 h. The silica was separated from the suspension by centrifugation. Absorption measurements on the supernatant at 280 nm gave an amount of 1.85 mg peptide in solution, which corresponds to an uptake of 1.15 mg (0.4 μmol) of melittin. Relative to the initial number of 3-mercaptopropyl moieties, the occupation of the potential binding sites for the melittin peptide can be calculated to be 60%. The SBA-MK particles were resuspended in 1.5 mL of phosphate-buffered saline (PBS; pH 7.4) and the resulting suspension was separated into two equal aliquots. One of the two resulting samples was centrifuged, the other one was diluted with 750 μL of PBS buffer (pH 7.4). The pellet of the centrifuged sample was resuspended in 1.5 mL PBS buffer (pH 5.5). The two resulting samples are designated as Quant-MK-7.4 and Quant-MK-5.5. After 60 minutes of incubation, the samples were centrifuged and the absorption of the supernatant was determined at 280 nm. It was found that the sample Quant-MK-5.5 had released 0.615 mg of melittin, while the sample Quant-MK-7.4 had only released 0.015 mg. The amount of released peptide corresponds well to the determined amount of incorporated melittin (a detailed calculation of the UV absorption using the Lambert–Beer law can be found in the Supporting Information).

The samples were used in an erythrocyte lysis assay to enable functional analysis of the released peptide. For this purpose, the suspensions of the samples SBA-BM-Mel and SBA-MK-Mel were serially diluted in PBS, which resulted in theoretical melittin concentrations of 2.0, 0.4, and 0.04 μM (calculated from the theoretical amount of peptide measured by absorption at 280 nm), respectively. Then, 75 μL of a mouse erythrocyte solution (3.75×10^6 cells) were mixed with 75 μL of the respective sample suspension or free melittin solution, which was used as a reference. The absorption at 405 nm of hemoglobin released from erythrocytes by freeze-thawing in PBS was set as 100% relative lysis. The acidic samples were preincubated at pH 5.5 for 60 minutes before dilution (a detailed description of the performed assay can be

found in the Supporting Information). For comparison, a standard curve of free melittin was produced. The results of the lysis assay are shown in Figure 3.

It can be seen that the MK linker shows the desired behavior and remains stable at pH 7, thereby preventing lysis

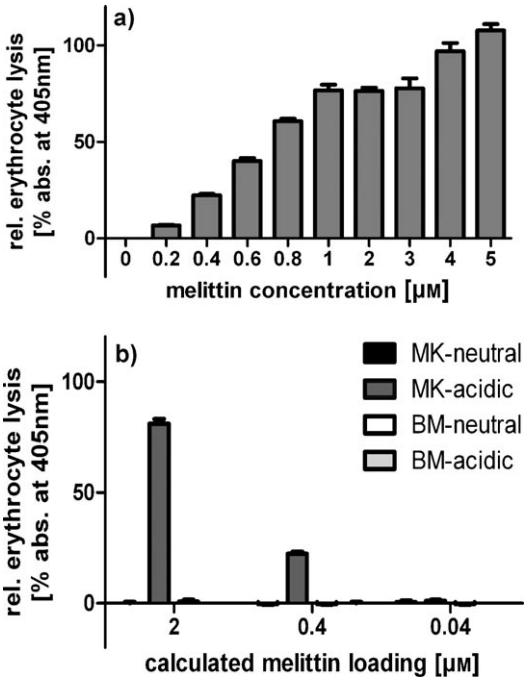

Figure 3. Results of the erythrocyte lysis assay (absorption of released hemoglobin at 405 nm relative to the value of erythrocytes lysed by freeze thawing). a) Relative erythrocyte lysis with different amounts of free melittin used as a standard. b) Relative erythrocyte lysis with SBA-MK-Mel and SBA-BM-Mel samples. The four experiments listed in the figure are plotted in same sequence from left to right at three different global concentrations calculated from absorption measurements at 280 nm.

even at the highest concentration tested. However, the MK linker is cleaved when the pH value is lowered, which results in a release of the encapsulated melittin. In contrast, the BM linker remains stable when the pH value is decreased, thereby keeping melittin covalently bound to the pore walls. The lytic activity of released melittin was quantitatively comparable to equal amounts of the free melittin standard. Thus, the lytic activity could be fully retrieved.

To summarize, we have shown that acetals can be applied as pH-sensitive linkers for the delivery of a biomedically relevant peptide from mesoporous silica. The obtained results represent an interesting alternative for the release of bioactive compounds from mesoporous hybrid materials. The results clearly show an on/off mechanism in the observed time intervals for the release. The investigated pH values represent the pH values in the human blood stream (7.4) and late endosomes in cells (5.5). This implies that the obtained results are of significant importance for the field of targeted drug delivery.

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