Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2020, **18**, 700

The role of phosphopeptides in the mineralisation of silica†

Fabian Daus, Erik Pfeifer, Kevin Seipp, Norbert Hampp and Armin Geyer *

Received 12th November 2019,
Accepted 18th December 2019

DOI: 10.1039/c9ob02438g

rsc.li/obc

We investigated the silicification activity of hyperphosphorylated peptides in combination with long-chain polyamines (LCPA). The bioinspired *in vitro* silicification experiments with peptides containing different amounts of phosphorylated serines showed structure–activity dependence by altering the amount and morphology of the silica precipitate. Our study provides an explanation for the considerable metabolic role of diatoms in the synthesis of hyperphosphorylated poly-cationic peptides such as natSil-1A$_1$. The efficient late-stage phosphorylation of peptides yielded a synthetic heptaphosphopeptide whose silicification properties resemble those of natSil-1A$_1$. As opposed to this, unphosphorylated poly-cationic peptides or LCPA require concentrations above 1 mM for silicification. Hyperphosphorylated peptides showed a linear dependence between the amount of dissolved peptides and the amount of precipitated silica in the concentration range below 1 mM. Under mildly acidic conditions and short precipitation times, the concentration of the added LCPA determined the size of the silica spheres.

Introduction

Diatoms are well known for the spectacular design of their species-specific nano- to micro-patterned silica-based exoskeletons.[1] Genetically controlled biomineralisation takes place in the silica deposition vesicle (SDV) in a mildly acidic environment.[2] Post-translational modification (PTM) results in enormous structural microheterogeneity of insoluble organic matrices which serve as scaffolds for biosilicification in the SDV.[3–5] Biosilica of diatoms is a composite material containing various organic compounds ranging from small soluble molecules and proteins to macromolecular organic matrices, and only some have been identified to date.[5–8]

Silaffins are the first proteins isolated from the biosilica of the marine diatom *Cylindrotheca fusiformis*.[7,8] These peptides are rich in serine and lysine and have quite similar sequences with a wide range of post-translational modifications.[7,8] Twelve out of 15 amino acids of the soluble peptide natSil-1A$_1$ show post-translational modifications. The lysine residues are methylated or alkylated with long-chain polyamines (LCPA) at the ε-amine position as well as hydroxylated and phosphorylated at the δ-position, and all seven serine residues of the peptide are phosphorylated.[8,9] This large number and diversity of PTM determines the unique bipolar poly-ionic character of natSil-1A$_1$.[9] However, natSil-1A$_1$ has become the role model peptide for *in vitro* silicification experiments due to its ability to precipitate silica from a mildly acidic solution at pH 5.5[2,8] (Fig. 1).

The investigation of biological systems is difficult due to the complexity of the sample. Quantification of structure–activity dependency requires the systematic stepwise introduction of individual PTM. The silicification properties of individual phosphopeptides may not correspond to the complex metabolic process of biomineralisation in diatoms. However, pushing the limits of *in vitro* silicification experiments to the high dilution range contributes to the understanding of the complementary behaviour of soluble cellular components involved in the mineralisation process, in contrast to other highly charged molecules such as the polyphosphate DNA and its associated polycationic proteins which are not involved.

Numerous bioinspired *in vitro* silica precipitation studies investigated the silicification properties of simplified synthetic analogs such as synthetic poly-cationic peptides lacking any PTM (R5 peptide),[10–14] peptides with LCPA-modified lysines,[15] and mono-phosphopeptides,[11] or of synthetic LCPA.[16] The silicification experiments with isolated natSil-1A or kinase re-phosphorylated proteins[17] are the only experiments with organic substrates bearing more than one covalently bound phosphate. The phosphorylation of the serine residues and the LCPA modifications have been proven to be essential for the silicification activity of silaffins under mildly acidic conditions.[2,7,8] A systematic stepwise variation in the number of bound phosphate esters to identify a minimal amount of phosphorylation has not been attempted yet. This is not only due to the lack of efficient experimental procedures for hyper-phosphorylation, but also because of the assumption that dis-

Department of Chemistry, Philipps-Universität Marburg, Hans-Meerwein-Straße 4, 35032 Marburg, Germany. E-mail: geyer@staff.uni-marburg.de

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ob02438g

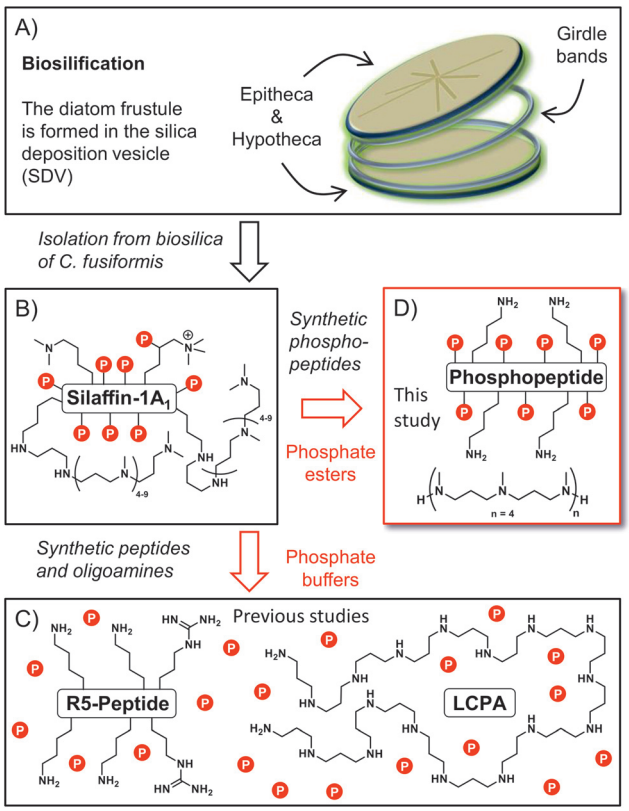


Fig. 1 (A) The morphogenesis of the diatom shell occurs in the silica deposition vesicle (SDV) during cell division. Dissolved silicic acid precipitates on a nanopatterned organic template in the presence of soluble components such as long-chain polyamines (LCPA) and peptides. (B) The poly-cationic phosphopeptide mixture natSil-1A was isolated from *Cylindrotheca fusiformis* by Kröger *et al.* in 2002.[8] (C) Numerous cationic peptides (left) or synthetic **LCPA** (right) were investigated for their silica precipitation activity in phosphate buffers or in the presence of other polybasic counterions. (D) In this work, the influence of the systematic increase of the degree of phosphorylation on the silicification process is investigated.

solved phosphate serving as a tribasic counterion for the polycationic organic substrate is sufficient for *in vitro* silica precipitation. Bioinspired *in vitro* silicification experiments focused on the amount and morphology of the obtained silica. For this purpose, high peptide concentrations and pH values were used to precipitate sufficient amounts of amorphous silica.

Results & discussion

We set out to synthesize natSil-1A$_1$ without PTM that we called **Sil01** and increased the degree of phosphorylation from one (**p1-Sil01**) to seven phosphate groups (**p7-Sil01**) with the aim of characterising the relevance of covalently bound phosphate. In order to achieve this, it was necessary to analyse the precipitation properties of unphosphorylated synthetic cationic peptides. Bioinspired *in vitro* silica precipitation methods have been carried out with synthetic silaffin peptides (**R5**) which precipitate silica from a phosphate buffered solution at a neutral pH value.[10–14] To investigate the influence of bound phosphate groups, we needed to perform an *in vitro* precipitation experiment without phosphate buffer and in a slightly acidic environment. The original study with natSil-1A isolated from *Cylindrotheca fusiformis* was performed in a 50 mM sodium acetate buffer under mildly acidic conditions at pH 5.5, with pre-hydrolysed TMOS solution as a silicic acid source.[8] In order to be able to compare our results with the study by Kröger *et al.*, we chose the same conditions as those of Kröger's study for our bioinspired *in vitro* silicification experiment. In addition to **Sil01**, we also investigated the **R5** peptide, because it has been usually studied in phosphate buffered solutions at neutral pH. The length distribution of natural LCPA was mimicked by a synthetic oligomer, which will be called **LCPA** in this study, synthesized according to Perry and co-workers[18] and is shown in Fig. 4. An average chain length of 12 nitrogen atoms was calculated from the integral ratio of the terminal and internal *N*-methyl groups (ESI†). It proved to be crucial to stop the silica precipitation after a defined time frame by the addition of 1.0 M HCl to obtain reproducible results.

In a mildly acidic environment (pH 5.5) in the absence of phosphate ions and **LCPA**, the poly-cationic peptides **Sil01** and **R5** show no silica precipitation activity, in the investigated concentration range. Millimolar peptide concentrations are necessary to precipitate silica in the presence of dissolved **LCPA** in sodium acetate buffer in a relevant time frame of a few minutes (Fig. 2). Isolated natSil-1A showed a linear dependence between the natSil-1A concentration and the amount of silica precipitate.[8] In contrast to the natural mixture natSil-1A, comprising natSil-1A$_1$ and natSil-1A$_2$, the lysine-rich peptides **Sil01** and **R5** require a threshold concentration above 1.2 mM,

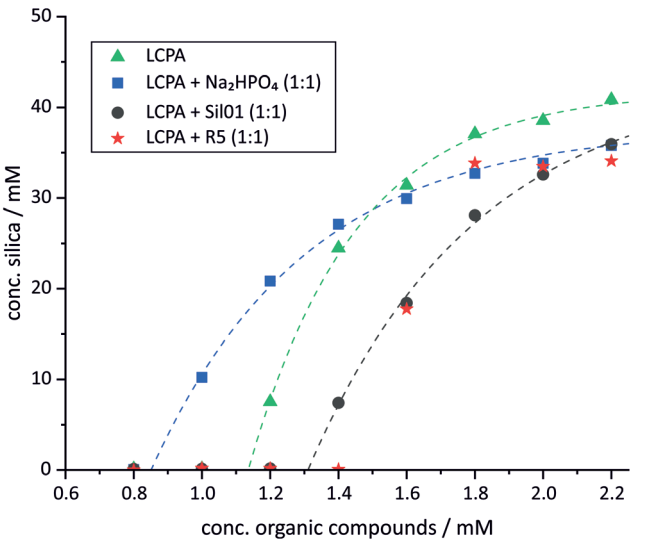


Fig. 2 Silica precipitation activity of different mixtures of **LCPA** and additives at pH 5.5 in a 50 mM sodium acetate buffer. The silica precipitation took place in a time frame of 10 min and was stopped by the addition of 1.0 M HCl. TMOS served as the source of silicic acid. Further experimental details are described in the ESI.†

below which no precipitated silica is observed (Fig. 2). The synthetic LCPA of this study closely resembles the distribution of chain lengths of the natural microheterogenic oligopropyleneamines in natSil-1A.

All three synthetic compounds, **LCPA**, **Sil01** and **R5**, show a quite similar dependence of concentration on precipitated silica. As the concentration of the organic compound increases, the amount of precipitated silica approaches the maximum value. Fig. 2 also shows that a minimum threshold concentration is required to induce the precipitation. The lowest threshold concentration was observed for an equimolar mixture of **LCPA** and sodium hydrogen phosphate. An analogous behaviour can be observed for an equimolar mixture of **LCPA** and **R5** peptides. However, due to the low number of data points, an asymptotic extrapolation of these data points is not reasonable.

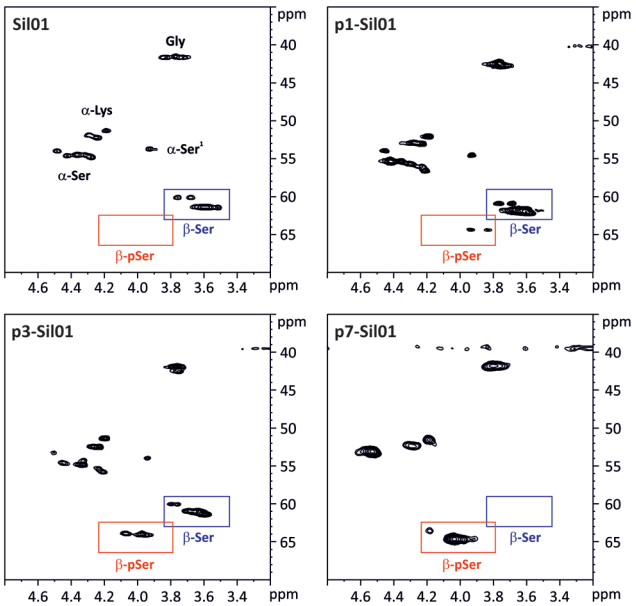


Fig. 3 HSQC spectra (600 MHz, 300 K, $DMSO_{d6}$) of the synthetic peptides. The blue boxes indicate the signals from the β-methylene groups of the non-phosphorylated serines and the red boxes indicate the β-methylene groups of the phosphorylated serines. The absence of β-methylene signals in the blue box at **p7-Sil01** indicates complete phosphorylation. The structures of the peptides are shown in Scheme 1.

Despite the routine incorporation of a single phosphoserine, a reliable and high-yielding multi-milligram hyperphosphorylation method together with a complete NMR spectroscopy analysis of the phosphopeptides has not been described yet. Dehydroalanine formation and incomplete phosphorylation are the drawbacks of synthetic phosphoserines. The uncharged side products are separable by HPLC for a monophosphopeptide but not for a heptaphosphopeptide, with over 5000 possible elimination products plus the same number of possible side products from phosphate ester hydrolysis. Just one percent of any side product would create an inseparable mixture. A heptaphosphopeptide described by Becker and co-workers was obtained in less than 1% yield, but this effort did not result in enough material for systematic silica precipitation experiments.[11] The efficient hyperphosphorylation of larger amounts of serine-rich peptides requires a quantitative phosphorylation step for each serine, since there is no possibility to separate the highly polar target peptides from incomplete phosphorylated species which elute together in the injection peak of the reversed-phase HPLC. Moreover, the dehydroalanine formation from the β-elimination reaction of protected phosphate esters leads to further inseparable side products. However, we found that it is impossible to separate the undesired lower oligophosphopeptides from the target peptides. Therefore, we focused on avoiding the formation of side products during synthesis in order to obtain sufficiently pure hyperphosphorylated peptides after resin cleavage.

One possible strategy for Fmoc-based solid phase phosphopeptide synthesis is the stepwise incorporation of a pre-phosphorylated amino acid derivative into the peptide chain. Another strategy is the post synthetic phosphorylation of a resin bound peptide (global phosphorylation). The major

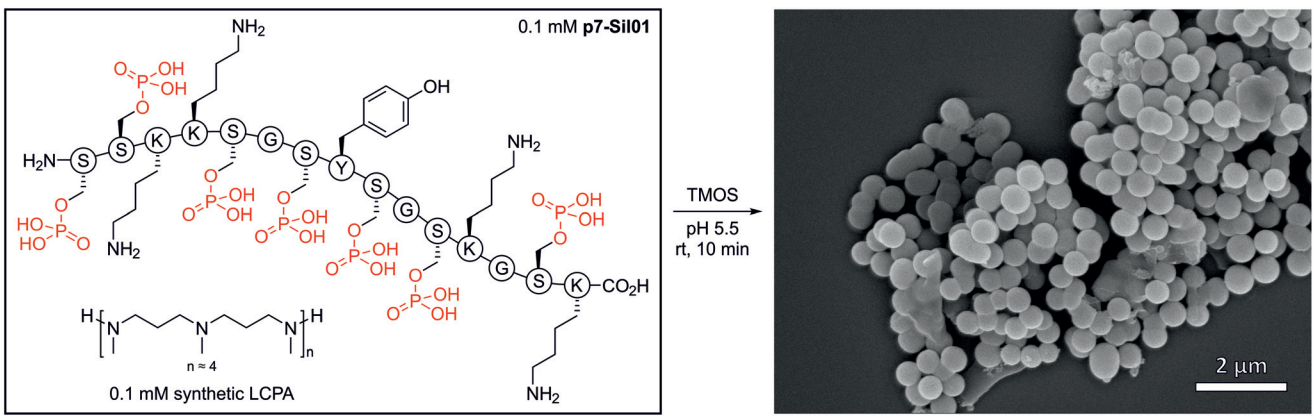


Fig. 4 Silica precipitation experiment of an equimolar mixture of **p7-Sil01** and **LCPA** led to silica spheres with an average diameter of 456 nm ± 52 nm. The precipitation experiment was performed at pH 5.5 in a 50 mM sodium acetate buffer with a peptide and **LCPA** concentration of 0.1 mM each, and the precipitation time was 10 min.

drawback of the stepwise incorporation of a pre-phosphorylated derivative is the piperidine-induced β-elimination during the removal of the Fmoc-group of dialkyl phosphoseryl derivatives.[19] In 1994, the introduction of monobenzylated derivatives (Fmoc-Ser(PO_3Bzl,H)-OH) to suppress β-elimination was a major step forward.[20] Optimised coupling and deprotection conditions allowed the complete suppression of β-elimination and provided peptides of high purity.[21] One or two phosphate esters were attached to the peptides using this method, but the stepwise incorporation appeared to be unsuitable for the synthesis of a heptaphosphopeptide such as **p7-Sil01**, where each of the seven critical coupling reactions depends on the individual stability of the already assembled phosphates of the lower homolog that is acylated on the resin.

Based on the above considerations, we decided to assemble the unphosphorylated, selectively mono-, tri- or heptahydroxy 15mer peptides as resin-linked precursors followed by exhaustive phosphorylation in a single step under systematically optimised reaction conditions. This approach required a global phosphitylation first, followed by oxidation to the desired phosphotriesters and finally chemoselective hydrolysis to serine monoesters.[19,22] The resin-bound peptide contained two different serine side chain protection groups of which the TBDMS ethers were selectively cleaved by TBAF treatment, to obtain a peptide with free serine hydroxy groups.[23] In order to avoid the final Fmoc-cleavage and thus possible β-elimination of the phosphotriesters, the N-terminal residue was Boc-protected.[19] Phosphoramidites in combination with an oxidising agent, *e.g.* *m*CPBA or ^{t}BuOOH, are the most common phosphorylation reagents.[24,25] In 1998, Perich showed that the amount of 1*H*-tetrazole used for the activation of the phosphoramidite is critical for the success of the overall synthesis. They showed that a large excess of 1*H*-tetrazole between 20 and 30 equiv. favours the formation of undesired *H*-phosphonates. This side reaction was further suppressed by the use of more stable dibenzyl-*N*,*N*-dialkylphosphoramidites under dilute conditions.[25] The optimised reaction conditions for the global phosphorylation are 3.0 equiv. of more stable phosphoramidite ($BnO)_2PN(i\text{-}Pr)_2$) and 1*H*-tetrazole in an excess up to a maximum of 7.5 equiv. in anhydrous DMF under an inert gas atmosphere with a final concentration of 0.2 M. The oxidation was carried out under dilute conditions with 37.7 equiv. of ^{t}BuOOH and a final concentration of 0.9 M in anhydrous DMF. With these optimised conditions, we obtained up to 100 mg of the heptaphosphorylated peptide in a single batch according to Scheme 1, which shows the solid-

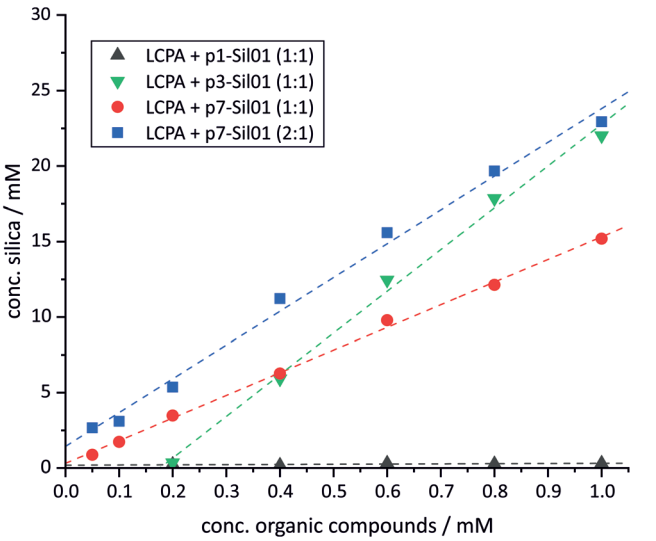
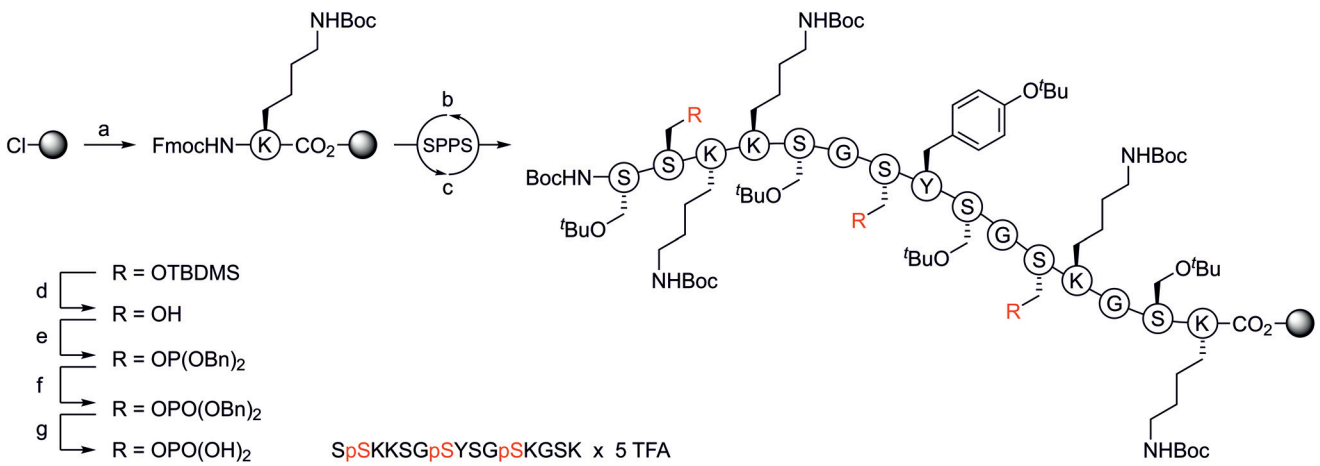


Fig. 5 Silica precipitation activity of mixtures of **LCPA** and phosphorylated derivatives of **Sil01** at pH 5.5 in a 50 mM sodium acetate buffer. The silica precipitation took place in a time frame of precisely 10 min and was stopped by the addition of 1.0 M HCl. TMOS served as the source of silicic acid. Further experimental details are described in the ESI.†



Scheme 1 SPPS and global late-stage phosphorylation of **p3-Sil01**. Monophosphorylated SSKKSGpSYSGSKGSK **p1-Sil01** and heptaphosphorylated pSpSKKpSGpSYpSGpSKGpSK **p7-Sil01** were synthesized accordingly by the incorporation of one or seven Fmoc-Ser(OTBDMS) building blocks. Reaction conditions: (a) Fmoc-Lys(Boc)-OH, DIPEA, DMF, rt, 180 min; (b) Fmoc-Aaa-OH, Oxyma, DIC, 50 °C, 35 W, 10 min; (c) 20% piperidine in DMF, 3 min; (d) TBAF, THF, rt, 60 min; (e) $(BnO)_2PN(i\text{-}Pr)_2$, 1*H*-tetrazole, DMF, rt, 180 min; (f) ^{t}BuOOH, decane, DMF, rt, 90 min; and (g) TFA, TIPS, H_2O, rt, 180 min.

phase protocol for the synthesis of a 15-mer resin-bound peptide.

The homogeneity of the peptides was characterised by NMR spectroscopy where the amount of elimination products, unphosphorylated serines or possible *H*-phosphonates is directly differentiated and quantified from the ^{1}H-NMR spectrum or from inverse *CH* correlations. As a bulk method, NMR is a reliable analytical method with high sensitivity, if a sufficient amount of peptide is available. HSQC spectroscopy verified the complete phosphorylation from the chemical shift variation of all serine methylene groups in **p7-Sil01** and the absence of unmodified primary hydroxyls or elimination products (Fig. 3). The overall signal dispersion and the chemical shifts showed little sensitivity to the number of phosphoser-

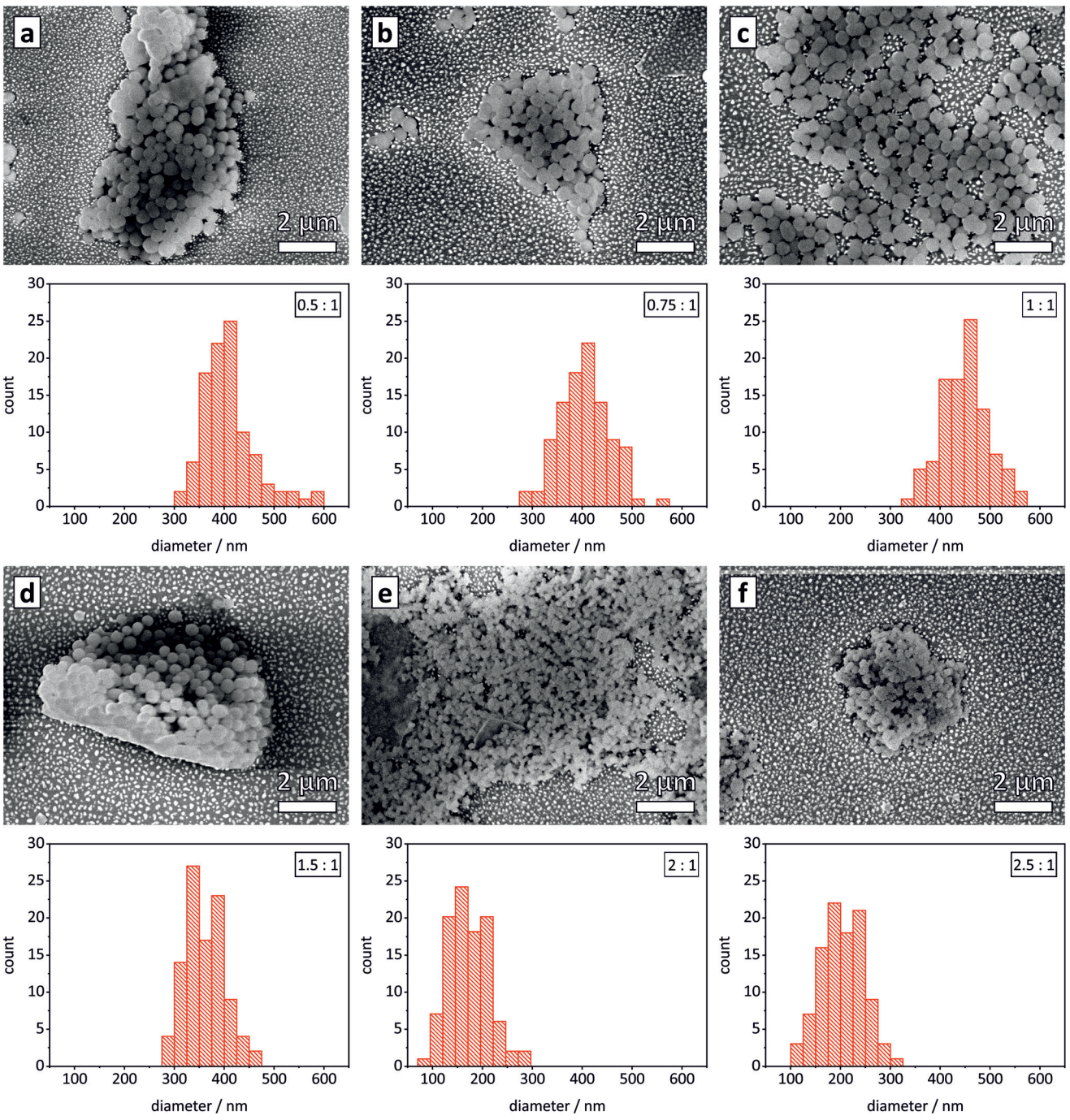


Fig. 6 SEM images (top) and histograms (bottom) of the diameter of the silica precipitates formed by the variation of the ratio between the **LCPA** and **p7-Sil01**. Boxes in the histograms (a) to (f) indicate the **LCPA** to **p7-Sil01** ratio of each silicification experiment. All the precipitates were obtained at pH 5.5 in a 50 mM sodium acetate buffer with a constant concentration (0.1 mM) of p7-Sil01. The bright dots surrounding the silica spheres are gold particles resulting from sample preparation ESI.†

ines and therefore a secondary structure formation or aggregation in DMSO is excluded.

The heptaphosphate **p7-Sil01** showed remarkable hydrolytic stability and remained unchanged even at pH 8 for more than one day. The silicification experiments with the phosphopeptides and with relevant reference compounds were performed according to the established procedures (ESI†).[8] It proved to be crucial to stop the silica precipitation after a defined time frame by the addition of 1.0 M HCl to observe the linear dependence between the concentration of organic compounds and the amount of precipitated silica. As expected, under mildly acidic conditions the phosphopeptides **p1-**, **p3-** and **p7-Sil01** show no silica precipitation activity in the absence of synthetic **LCPA**. The LCPA modifications have been proven to be essential for the activity of silaffins under mildly acidic conditions.[7]

In comparison with unphosphorylated **Sil01**, the monophosphorylated **p1-Sil01** has no influence on the silica precipitation properties. Therefore, no silica precipitate is observed below 1.0 mM concentrations (Fig. 5). The triphosphate **p3-Sil01** showed a significantly lower threshold concentration of around 0.2 mM, at which silica precipitation started. However, the heptaphosphate **p7-Sil01** precipitated silica even at 0.05 mM with a linear dependence of the peptide concentration on the amount of silica precipitate. A threshold concentration for **p7-Sil01** at which the silica precipitation is initiated was not observed within the investigated concentration range. This behaviour suggests that synthetic **p7-Sil01** has a very low threshold concentration at which the silica precipitation is initiated. Fig. 5 shows that the amount of **LCPA** has a direct influence on the amount of silica precipitate. A molar ratio of 15 : 1 of silica precipitate and **p7-Sil01** was observed for an equimolar mixture of **LCPA** and **p7-Sil01**. This ratio increases to 22 : 1 when the amount of **LCPA** is doubled. This linear dependence is of specific interest since **p7-Sil01** is the only synthetic compound which exhibits such a linear dependence at high dilution and under mildly acidic conditions which represents the relevant pH range of the SDV. A similar linear dependence was described only for the isolated natSil-1A by Kröger *et al.* in a concentration range from 0.1 mM to 0.5 mM.[8]

Based on the influence of the amount of **LCPA**, we conducted further experiments with different **LCPA** to **p7-Sil01** ratios. Fig. 6 shows the silica precipitates for the **LCPA** to **p7-Sil01** ratios between 0.5 : 1 and 2.5 : 1. The distribution of sphere sizes is very homogeneous for each ratio with an average bead diameter of around 400 nm up to a ratio of 1 : 1 and a significantly smaller diameter of around 200 nm for ratios higher than 1 : 1.

The size of the silica spheres depends on the amount of **LCPA** and the average bead diameter changes significantly when the **LCPA** to peptide ratio is increased above 2 : 1 (Fig. 6 and 7). The largest silica spheres with an average diameter of 456 nm ± 52 nm were obtained from an equimolar mixture of **LCPA** and **p7-Sil01**. With decreasing size, the formerly separated silica spheres fuse together, generating agglomerates. The morphology of the silica spheres which were obtained for the equimolar mixture of **LCPA** and **p7-Sil01** closely resembles that of the silica spheres obtained from natSil-1A, as reported by Kröger *et al.*[8]

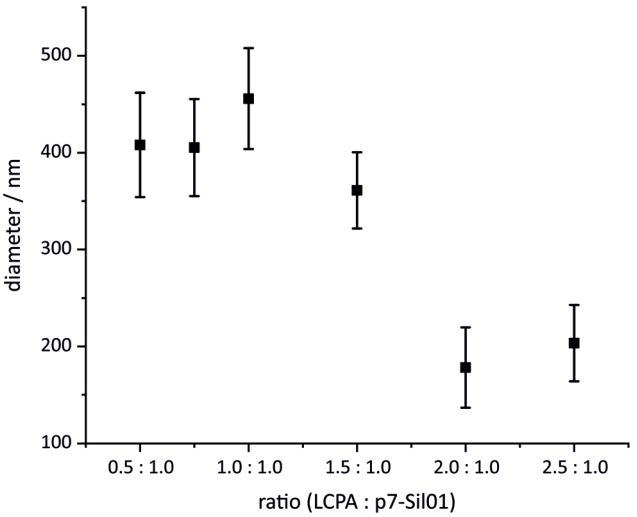


Fig. 7 **LCPA** to **p7-Sil01** ratio in dependence to the average diameters of the obtained silica spheres.

Biosilicification of diatoms starts with the formation of siloxane bonds, followed by aggregation of silica nanospheres and deposition onto micrometre-sized structures by the scaffolding effects of organic matrices.[3,26] The *in vitro* experiments of this study show that phosphopeptides together with LCPA precipitate silica in the absence of organic matrices. We assume that poly-anionic and poly-cationic components form a polyelectrolyte complex stabilized by electrostatic interactions, a concept well established in polymer science.[27] Polyelectrolyte complexes are plausible nucleation sites which initiate the silicification process and explain the significantly higher silicification activity of hyperphosphorylated peptides (Fig. 5) compared to unphosphorylated peptides (Fig. 2) at low concentrations, because of the strong association tendency of highly complementary charged ions. The heptaphosphorylated **p7-Sil01** is the only synthetic compound that shows a linear dependence between the peptide concentration and the amount of silica precipitate even at high dilutions. This linear behaviour was previously observed only in isolated natSil-1A. A linear dependence between the peptide concentration and the amount of silica is well-known in silicifying enzymes such as glassin which catalyse the siloxane bond formation.[28] Hyperphosphorylated peptides are distinctively different from lysine-rich peptides, which mediate silica precipitation only at higher concentrations in phosphate buffers or at neutral pH (Fig. 2). A significant precipitation activity at low concentrations is a prerequisite for a site-specific silica precipitation.

Conclusion

In conclusion, we described an efficient protocol for the synthesis of phosphorylated peptides containing up to seven monophosphate esters. These synthetic phosphopeptides exhibit a significantly improved silicification activity at low

concentrations and pH values in the absence of phosphate buffers compared to the standard arginine- or lysine-rich peptides in phosphate buffers. In conjunction with variable amounts of **LCPA**, the heptaphosphopeptide **p7-Sil01** shows silicification activity down to 0.05 mM while retaining the silica morphology observed for the isolated natSil-1A.

Experimental

The methods (including silica precipitation assay and SEM measurements), details of synthesis (including peptide synthesis and phosphorylation) and the characterisation of compounds (including NMR, HRMS and HPLC) are described in the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors would like to thank the members of the Nanomee research group (FOR 2038) for their helpful discussions and the Deutsche Forschungsgemeinschaft (DFG) for financial support.

References

1 (*a*) E. V. Armbrust, *Nature*, 2009, **459**, 185–192; (*b*) F. E. Round, R. M. Crawford and D. G. Mann, *The diatoms. Biology & morphology of the genera*, Cambridge Univ. Pr, Cambridge, 1990.
2 E. G. Vrieling, W. W. C. Gieskes and T. P. M. Beelen, *J. Phycol.*, 1999, **35**, 548–559.
3 N. Kröger, *Curr. Opin. Chem. Biol.*, 2007, **11**, 662–669.
4 (*a*) B. Tesson and M. Hildebrand, *PLoS One*, 2013, **8**, e61675; (*b*) M. Hildebrand, S. J. L. Lerch and R. P. Shrestha, *Front. Mar. Sci.*, 2018, **5**, 125.
5 A. Scheffel, N. Poulsen, S. Shian and N. Kröger, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 3175–3180.
6 (*a*) M. Sumper, E. Brunner and G. Lehmann, *FEBS Lett.*, 2005, **579**, 3765–3769; (*b*) N. Kröger, R. Deutzmann, C. Bergsdorf and M. Sumper, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 14133–14138; (*c*) E. Brunner, P. Richthammer, H. Ehrlich, S. Paasch, P. Simon, S. Ueberlein and K.-H. van Pée, *Angew. Chem., Int. Ed.*, 2009, **48**, 9724–9727; (*d*) N. Kröger, C. Bergsdorf and M. Sumper, *EMBO J.*, 1994, **13**, 4676–4683; (*e*) N. Kröger, C. Bergsdorf and M. Sumper, *Eur. J. Biochem.*, 1996, **239**, 259–264; (*f*) N. Kröger, G. Lehmann, R. Rachel and M. Sumper, *Eur. J. Biochem.*, 1997, **250**, 99–105; (*g*) N. Kröger and R. Wetherbee, *Protist*, 2000, **151**, 263–273; (*h*) S. Wenzl, R. Hett, P. Richthammer and M. Sumper, *Angew. Chem., Int. Ed.*, 2008, **47**, 1729–1732; (*i*) P. Richthammer, M. Börmel, E. Brunner and K.-H. van Pée, *ChemBioChem*, 2011, **12**, 1362–1366.
7 N. Kröger, R. Deutzmann and M. Sumper, *Science*, 1999, **286**, 1129–1132.
8 N. Kröger, S. Lorenz, E. Brunner and M. Sumper, *Science*, 2002, **298**, 584–586.
9 N. Kröger, R. Deutzmann and M. Sumper, *J. Biol. Chem.*, 2001, **276**, 26066–26070.
10 M. R. Knecht and D. W. Wright, *Chem. Commun.*, 2003, **24**, 3038–3039.
11 C. C. Lechner and C. F. W. Becker, *Chem. Sci.*, 2012, **3**, 3500–3504.
12 L. Senior, M. P. Crump, C. Williams, P. J. Booth, S. Mann, A. W. Perriman and P. Curnow, *J. Mater. Chem. B*, 2015, **3**, 2607–2614.
13 E. L. Buckle, A. Roehrich, B. Vandermoon and G. P. Drobny, *Langmuir*, 2017, **33**, 10517–10524.
14 C. C. Lechner and C. F. W. Becker, *J. Pept. Sci.*, 2014, **20**, 152–158.
15 R. Wieneke, A. Bernecker, R. Riedel, M. Sumper, C. Steinem and A. Geyer, *Org. Biomol. Chem.*, 2011, **9**, 5482–5486.
16 A. Bernecker, R. Wieneke, R. Riedel, M. Seibt, A. Geyer and C. Steinem, *J. Am. Chem. Soc.*, 2010, **132**, 1023–1031.
17 V. Sheppard, N. Poulsen and N. Kröger, *J. Biol. Chem.*, 2010, **285**, 1166–1176.
18 V. V. Annenkov, S. N. Zelinskiy, E. N. Danilovtseva and C. C. Perry, *ARKIVOC*, 2009, **13**, 116–130.
19 L. Otvos, I. Elekes and V. M. Lee, *Int. J. Pept. Protein Res.*, 1989, **34**, 129–133.
20 T. Wakamiya, K. Saruta, J.-i. Yasuoka and S. Kusumoto, *Chem. Lett.*, 1994, **23**, 1099–1102.
21 (*a*) J. W. Perich, N. J. Ede, S. Eagle and A. M. Bray, *Lett. Pept. Sci.*, 1999, **6**, 91–97; (*b*) T. J. Attard, N. M. O'Brien-Simpson and E. C. Reynolds, *Int. J. Pept. Res. Ther.*, 2009, **15**, 69–79.
22 D. M. Andrews, J. Kitchin and P. W. Seale, *Int. J. Pept. Protein Res.*, 1991, **38**, 469–475.
23 C. E. Schumacher, P. W. R. Harris, X.-B. Ding, B. Krause, T. H. Wright, G. M. Cook, D. P. Furkert and M. A. Brimble, *Org. Biomol. Chem.*, 2017, **15**, 8755–8760.
24 E. A. Ottinger, L. L. Shekels, D. A. Bernlohr and G. Barany, *Biochemistry*, 1993, **32**, 4354–4361.
25 J. W. Perich, *Lett. Pept. Sci.*, 1998, **5**, 49–55.
26 S. V. Patwardhan, S. J. Clarson and C. C. Perry, *Chem. Commun.*, 2005, 1113–1121.
27 L. Chiappisi, I. Hoffmann and M. Gradzielski, *Soft Matter*, 2013, **9**, 3896.
28 K. Shimizu, T. Amano, M. R. Bari, J. C. Weaver, J. Arima and N. Mori, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 11449–11454.

