

Identification of Binding Peptides on Calcium Silicate Hydrate: A Novel View on Cement Additives

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Cement is the most used industrial product in the world. Although the chemical composition of the material has stayed more or less the same since its discovery by the Romans around 2000 years ago,^[1] the performance has been increased by chemical additives. Spectacular buildings like the Willis Tower in Chicago, Taipei 101 or lately the over 800 m high Burj Khalifa in Dubai were realizable thanks to the development of high performance building materials.^[2] Not only for such prestige objects but also in daily building processes, the trend goes towards always higher buildings because of the continued urbanization which was identified already in 1982 as one of the so-called “megatrends”.^[3] Therefore, the requirements for building materials are getting more and more demanding. Industry tries to face this challenge with sophisticated products that modify chemical or physical properties of cement according to the desired properties. Such products can for example be superplasticizers (e.g. polycarboxylate ethers), which improve the viscosity or inorganic chemical additives modifying the hydration and/or the development of mechanical properties.

In this study, we focus on calcium silicate hydrate (C-S-H),^[4] a compound with variable Ca/Si ratio depending on the pH and the calcium concentration in solution. It forms during the hydration of anhydrous calcium silicate phases which are the predominant components of ordinary Portland cement. It is generally accepted that C-S-H is the main source of cohesion in cement and thus, responsible for its hardening and setting.^[5] Due to its key role for the final performance of mortars and concrete, it was already tried to change and improve its properties with organic additives.^[6–12] Up to now, the focus was only on cationic^[6–8,13] or anionic^[7,9,12,14] polymers with high charge densities. However, in multi component systems like cement, where a large panoply of different compounds is present during the hydration process, suitable polymers have to be identified which are capable to undergo strong and particularly specific adsorption on C-S-H. Current commercial additives like polycarboxylate ethers mainly address Coulomb attraction^[15] while strong and particularly specific adsorption may be facilitated

by interactions going beyond pure electrostatics. To shed light on the best binding motifs, which could constitute an optimal additive, we performed a phage display (PD) on C-S-H in equilibrium with different calcium hydroxide concentrations giving different surface properties. PD is a common selection method in biology that uses genetically modified bacteriophages for the identification of strong binding peptides on substrates.^[16] Many publications deal with the specific binding of peptides on inorganic materials like semiconductors,^[17–20] minerals,^[21–23] platinum,^[24] gold,^[25] etc. Despite numerous published studies with silicon dioxide,^[26–29] C-S-H has not been investigated yet. Moreover, none of these studies takes place at highly alkaline pH values that are necessary for C-S-H stability, but always at physiological conditions.

For this study focusing on additive – substrate interactions, the surface chemistry of C-S-H is particularly important (**Figure 1**). Due to silanol (Si-OH) functions and their partially deprotonated Si-O⁻ groups on the surfaces (depending on pH), C-S-H particles are highly negatively charged at elevated pH values.^[30] In the presence of divalent cations like Ca²⁺, electrostatically based attractive forces can be observed between these surfaces (caused by ionic correlation effects),^[31] eventually leading to an inversion of the zeta potential (overcharging effect).^[32] These forces between C-S-H particles are short ranged (1–3 nm),^[33] which is the main reason for the low maximal elastic deformation of cement materials (~0.03%).^[34]

With aqueous C-S-H suspensions being highly alkaline with pH values up to 12.5, the pH stability and activity of the bacteriophages at these conditions must be ensured. To this end, reference experiments in highly basic media were performed. Results suggest that the conditions during the panning experiments do not have to be physiological (Figure S1). Up to pH 12, nearly all phages stay stable and active. Although their amount is dramatically decreased for pH values >12 this outcome paves the way of the method to other alkaline systems.

In view of these findings, C-S-H as substrate is accessible for the phage display method. **Table 1** shows the identified peptide sequences for C-S-H in equilibrium with a solution of the lowest pH, noted by C-S-H 0.66 which refers to the calcium to silicon ratio of the initial lime-silica mix. The pH during the panning experiment was 8.9 (due to the addition of phages, stored in TBS (tris-buffered saline), Table S2). XRD measurements after exposure of C-S-H 0.66 to those conditions still confirmed the presence of C-S-H which might have dissolved at such a low pH value (Figure S2). At pH 8.9 both alkaline amino acids lysine (K) and arginine (R) are positively charged (Figure S3) and with 35% they are the predominant species in the sequences (grey highlighted in Table 1). Arginine was enriched by a factor of 2.9, lysine even by a factor of 6 compared to the distribution of amino acids in the original phage

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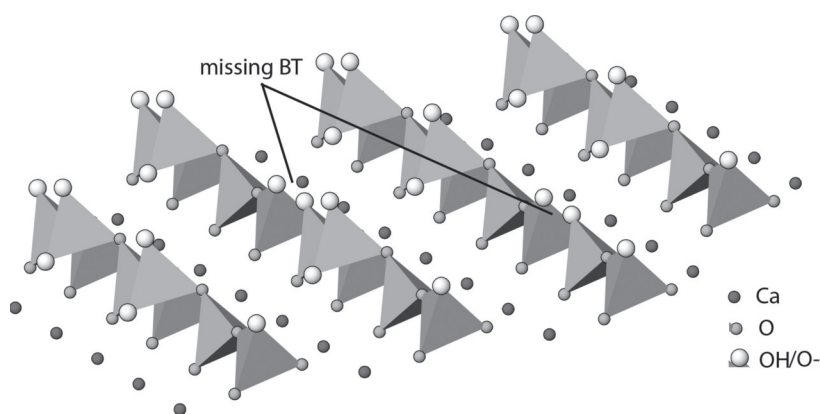


Figure 1. Structure of the C-S-H surface. C-S-H as a layered structure constituted by a double plane of calcium cations between two identical planes consisting of parallel linear chains of silicate tetrahedra. Silicate chains have a “dreiketten” structure, i.e. a repetition of three tetrahedra, one dimer and a bridging tetrahedron (BT) ensuring the connection with the following dreierkette. Above, the silicate plane which constitutes the surface is represented: depending on the pH, the non-bonded oxygen atoms of silanol groups SiOH are more or less deprotonated conferring negative charges to the surface which are compensated and even overcompensated in high calcium hydroxide concentration, leading to an apparent positive charge. Beside the deprotonation of silanol groups, the increase of the pH also depolymerises the silicate chains by eliminating parts of the bridging. The disappearance of bridging tetrahedra does not have an effect on the surface SiOH density which is 4.8 SiOH/nm². The surface properties of the C-S-H samples considered in this study are reported in Table S1.

display library (Table S3). The isoelectric points (pI) of the peptides with free N- and C-terminus were determined in a range of 9.7 – 11.5,^[35] the weighted average hydrophilicity to $+0.94 \pm 0.41$ (calculated as described elsewhere).^[36] In essence, this finding can be understood taking the structure of C-S-H 0.66 into account (Figure 1 and Table S1). Zeta potential measurements ($\zeta \approx -8$ mV, pH 10.3) as well as Monte Carlo simulations revealed, that its surface is covered with silanol groups that are partially deprotonated and poorly compensated in its close vicinity by Ca²⁺-ions because of a comparably low charge density of C-S-H 0.66 under the given conditions.^[32] Thus, electrostatic interactions between deprotonated silanol groups and positively charged amino acids are favored.

Adsorption experiments of synthesized peptides on C-S-H were performed on C-S-H 0.66 to better understand the

Table 1. Identified sequences for C-S-H 0.66 at pH 8.9. Positively charged amino acids at the respective pH are highlighted in grey.

Name	Identified sequences	Quantity [%]	pI	Hydrophilicity ^[18]
0.66_1	K P P K R K A D R W V P	30	11.3	1.1
0.66_2	L G A D R T R D R R H N	20	11.5	1.3
0.66_3	T E R R G S S K Y P K R	15	11.1	1.3
0.66_4	S H L P K V R S P T Q K	10	11.4	0.5
0.66_5	N K W P L A H S Q K K R	10	11.5	0.5
0.66_6	L K P N K P R M H L D I	5	10.4	0.4
0.66_7	G G S G T S R T P I L G	5	9.7	-0.1
0.66_8	S R S S G L K K Q Y H K	5	10.7	0.7
weighted mean value			11.2 ± 0.4	0.94 ± 0.41

role and nature of favored positive charge (Table 2). According to this, two features have to be considered for strong adsorption on C-S-H 0.66:

- (1) Longer chain lengths are more favored for adsorption than shorter ones (displayed by the much higher adsorption rates for 0.66_1a and 0.66_2a compared to 0.66_1 and 0.66_2, respectively).
- (2) Homogeneously distributed charge is favored over local high charge densities (Var_1 vs. Var_2). However, best rates are obtained from purely positively charged residues. Again, the longer the chain length the better the adsorption rate (Var_3 vs. Var_3a). Consequently, best adsorption was observed for polylysine (Var_11), where nearly 95% bound to C-S-H 0.66. These results are fully consistent with recent experiments and Monte Carlo simulations of oligocation adsorption on C-S-H.^[13]

When the equilibrium calcium hydroxide concentration is increased to 2.3 mM (C-S-H 1.0), two effects being responsible for the electrostatic dominance are reduced. Due to the higher concentration of Ca(OH)₂ in the equilibrium solution, the pH is raised up to 12 (11.6 during the biopanning), so that at least lysine is neutral while arginine keeps an average net charge of around +0.7 (Figure S3). The second effect is related to the surface of C-S-H, which is more negatively charged and partly covered by Ca²⁺ ions due to the elevated calcium concentration (Figure 1 and Table S1). The negative charge of the deprotonated silanol groups is electrostatically shielded so that charge effects between bacteriophages and C-S-H are reduced. Zeta potential measurements of C-S-H 1.0 with a value around 0 mV (pH 12.0) as well as Monte-Carlo simulations^[32] underline this

Table 2. Adsorption of chosen identified sequences from the PD experiments and amino acid variations on C-S-H 0.66. Positively charged amino acids are highlighted in grey, negatively charged ones in black.

Name	Sequence	Adsorbed on C-S-H 0.66 [%]
0.66_1	K P P K R K A D R W V P	59.3
0.66_1a	2x 0.66_1	75.0
0.66_2	L G A D R T R D R R H N	18.2
0.66_2a	2x 0.66_2	46.0
Var_1	R K R A G A R K R A G A	59.1
Var_2	K A K A K A K A K A K A	65.3
Var_3	K K K K K K K K K K K K	69.3
Var_3a	2x Var_3	71.3
Var_11	Poly Lys (111 – 177)	94.4
Var_12	Poly Asp (18 – 26)	37.2

assumption. This is furthermore confirmed by calculations of the hydrophilicities of the identified peptides (Table 3a), which are dramatically decreased from 0.94 ± 0.41 (C-S-H 0.66) to an average value of -0.08 ± 0.26 . Purely electrostatic interactions between the C-S-H surface and charged, hydrophilic amino acids do not seem to play such a dominating role anymore. The isoelectric points (4.6–9.7) suggest, that also slightly acidic peptides are taken into account during the selection process (Table 3a). Moreover, nearly 50% of the identified amino acids are capable to undergo H-bond formation while only 14% are charged. The rest of the sequences consists of hydrophobic amino acids (Table 4).

Although the amount of negatively charged amino acids is slightly increased for C-S-H 1.5 (C-S-H in equilibrium with a saturated calcium hydroxide solution) due to the overcompensation by calcium ions giving an increased zeta potential of $\approx +20$ mV^[32] (pH 12.5), the overall tendency stays the same as for C-S-H 1.0. H-bond forming amino acids make up around 1/3 of the identified residues, while the share of hydrophobic amino acids even increased to $\approx 50\%$ (Table 4). Here, the pI of the identified peptides for both C-S-H 1.5 and C-S-H 1.7 (same C-S-H as C-S-H 1.5 with an excess of calcium hydroxide) ranges between 4.3 and 9.7 while the average hydrophilicities decrease only slightly to -0.14 ± 0.21 and -0.18 ± 0.17 , respectively (Table 3b and 3c). This data suggests that the identified peptides are not only adsorbed because of their strong Ca^{2+} mediated electrostatic interactions with the C-S-H surface, but also due to their specific binding motifs which include H-bond formers and hydrophobic residues.

“One feature can be determined in nearly all identified sequences for C-S-H 1.0 to 1.7. 79% of all bound peptides on C-S-H 1.0 and 1.7 and even 94% of C-S-H 1.5 bear at least one negatively charged amino acid like aspartic acid (D), glutamic acid (E) or tyrosine (Y) (red highlighted in Tables 3a–c) in combination with two to nine H-donating groups like arginine (R), lysine (K), serine (S), asparagine (N) etc. (green highlighted in Tables 3a–c).” Taking again the surface structure of C-S-H into account (Figure 1 and Table S1), this panoply of identified interactions seems to be quite reasonable. We assume that negatively charged amino acids interact via electrostatic interactions with the Ca^{2+} -ions which are adsorbed on surface Si-O^- groups whereas non charged amino acids can bind via H-bonds (e.g. R, K, S) and additional Ca^{2+} mediated adsorption (e.g. Asn with the amide group bearing a partial negative charge on the carbonyl oxygen) as proposed in Scheme 1. The combination of these types of interactions results then in strong and particularly specific adsorption. Although the Ca^{2+} binding capability of single amino acids has not been investigated yet, previous studies with polyaspartic acid claim that pAsp is dependent on its molecular weight, able to bind up to 0.29 Ca^{2+} ions per monomer at pH 9.75 ($M_w = 27000$ g/mol).^[37] Also the adsorption capability of amides like polyacrylamide is known for silicate minerals like kaolinite^[38] so that the proposed interaction model seems to be conclusive.

Most surprisingly is the predominance of Pep E (identified to 64% for C-S-H 1.5 and 24% for C-S-H 1.7). With its 3 leucine (L) and one isoleucine (I), it shows a very high hydrophobicity in addition to the other features discussed above (H-bonds +

amide group + negative charge). It displays hydrophobic-hydrophilic motif (HLGAIL – RAD) with a bulky hydrophobic block (LGAIL). This additional property can make the difference to the remaining binding peptides and might be responsible for an even higher adsorption capacity on the substrate. To elucidate the role of the different parts of Pep E on the binding, further adsorption experiments on C-S-H were performed with Pep E and some amino acid variations (Table 5).

Var_4 – Var_6 are variations of the RAD-motif of Pep E with different spacings between R and D and R and E. While adsorption rates get higher without spacing groups for C-S-H 1.5 (Var_4 > Var_5 > Var_6), they decrease in case of C-S-H 1.0 (Var_6 > Var_5 > Var_4), suggesting a folding/collapse of the peptides upon interaction with Ca^{2+} getting more pronounced for C-S-H 1.5, which contains more Ca^{2+} than C-S-H 1.0 thus resulting in lower adsorption. For the smallest spacing between R and D, Ca^{2+} interaction is partly hindered by R and the peptide folding/collapse is hindered so that the adsorption of Var_4 is higher for C-S-H 1.5 than C-S-H 1.0. When the spacing between R and D becomes bigger, Ca^{2+} interaction is increased, the peptides fold/collapse more for C-S-H 1.5 resulting in a smaller adsorption. The folding/collapse of the peptide upon Ca^{2+} interaction is confirmed by Var_7, which only contains D with an uncharged spacer and can thus collapse and interact less with C-S-H 1.5 than C-S-H 1.0. Thus, negative charge alone (Var_7) shows the lowest adsorption rates and confirms the insufficiency of this motif at these conditions. Only by increasing the chain length, binding of anionic species gets more efficient (Var_12). However, this was already shown before to increase the adsorption rate of all peptides. Samples Var_8 – Var_10 show variations of the hydrophobic – hydrophilic motif from Pep E. Bearing no single charged amino acid, they exhibit even higher adsorption rates as Var_4 – Var_6. Var_8 (only bearing H-bond formers, one amide function and hydrophobic residues after the exchange of RAD to SAS) is adsorbed much more (nearly by a factor of 2 for C-S-H 1.0) on C-S-H than Pep E. The same is true for Var_9 with its well distributed alternating hydrophobic – hydrophilic motif, while huge blocks appear to be less bound (Var_10). The results underline again, that hydrophobic interactions in combination with H-donating and amide functions are more favored for specific adsorption on C-S-H than completely charged additives.

In conclusion, our findings show that a strong and particularly specific adsorbing organic additive on industrially important C-S-H should have three different features:

- 1: one negatively charged part which adsorbs on Ca^{2+}
- 2: H-donating or amide functions for (Ca^{2+} mediated) interactions with deprotonated silanol groups
- 3: a highly hydrophobic part

This outcome suggests a hypothetical interaction model as shown in Scheme 1, which differs very much from hitherto applied polyelectrolyte cement additives adsorbing mainly via electrostatic interactions. Our results clearly show that the adsorption of additives on C-S-H at pH < 11 is mainly dominated and driven by electrostatic interactions only, while combinations of charge with hydrophobic interactions and

Table 3. Identified sequences for C-S-H 1.0, 1.5 and 1.7. Positively charged amino acids at the respective pH are highlighted in grey, negatively charged ones in black and H-donating groups in light grey. Arginine is protonated to only $\approx 25\%$ at pH 12.5 and hence shaded in b and c.

a) C-S-H 1.0, pH 11.6

Name	Identified sequences	Quantity [%]	pI	Hydrophilicity ^[18]
Pep A	Y H P N G M N P Y T K A	26	8.4	-0.3
1.0_1	L P G R A H D P W K V P	21	8.9	0.1
Pep B	S A T N G S L T R P V H	16	9.7	-0.1
Pep C	G T T T L N H N Y S A K	11	8.5	-0.2
1.0_2	D G N T A A R M A T L K	11	8.9	0.3
Pep D	V P R S M A A T H S T F	5	9.7	-0.3
1.0_3	A N H L S G N N Y G I S	5	6.8	-0.5
1.0_4	D S A S T Q F T R A D S	5	4.6	0.5
weighted mean value			8.6 ± 1.1	-0.08 ± 0.26

b) C-S-H 1.5, pH 12.5

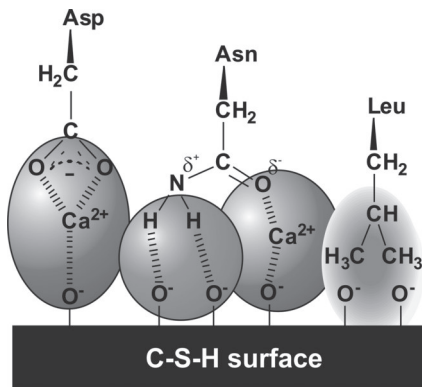
Name	Identified sequences	Quantity [%]	pI	Hydrophilicity ^[18]
Pep E	L P N H L G A I L R A D	64	6.8	-0.2
1.5_1	S S L H S S H H E M N K	6	7.1	0.2
Pep C	G T T T L N H N Y S A K	3	8.5	-0.2
1.5_2	K F N P G N S E W Q R T	3	8.9	0.3
1.5_3	S K H D T P T Y I N T V	3	6.8	-0.1
1.5_4	I D R V T S R D P A M N	3	6.1	0.6
1.5_5	V D T V K H E L A T Y R	3	6.8	0.3
Pep B	S A T N G S L T R P V H	3	9.7	-0.1
Pep A	Y H P N G M N P Y T K A	3	8.4	-0.3
Pep D	V P R S M A A T H S T F	3	9.7	-0.3
Pep F	G G S I A A S E L E Y Y	3	4.3	-0.2
1.5_6	I F A A L D Y N L G R H	3	6.8	-0.5
weighted mean value			7.1 ± 1.0	-0.14 ± 0.21

c) C-S-H 1.7, pH 12.5

Name	Identified sequences	Quantity [%]	pI	Hydrophilicity ^[18]
Pep E	L P N H L G A I L R A D	24	6.8	-0.2
Pep A	Y H P N G M N P Y T K A	24	8.4	-0.3
Pep F	G G S I A A S E L E Y Y	17	4.3	-0.2
Pep B	S A T N G S L T R P V H	7	9.7	-0.1
1.7_1	T L R V P P N P N M N V	7	9.7	-0.2
Pep D	V P R S M A A T H S T F	3.5	9.7	-0.3
1.7_2	I E A Q Y N H S N R F P	3.5	6.8	0.1
1.7_3	M T F D T V S N I Y K M	3.5	6.0	-0.4
1.7_4	H A A G I R D N Q R L G	3.5	9.5	0.4
1.7_5	D G N T A A R M A T L K	3.5	8.9	0.3
1.7_6	T C A K A T S T P P L S	3.5	8.3	-0.1
weighted mean value			7.8 ± 3.8	-0.18 ± 0.17

Table 4. Groups of identified amino acids by phage display at various conditions.

Sample	Negatively charged amino acids [%]	Positively charged amino acids [%]	Amino acids for H-bond formation [%]	Hydrophobic amino acids [%]
C-S-H 0.66	7.5	34.6	26.2	31.7
C-S-H 1.0	9.2	4.8	47.4	38.6
C-S-H 1.5	10.1	7.1	28.6	54.2
C-S-H 1.7	13.1	4.6	35.2	47.1



Scheme 1. Proposed interaction model of the chosen amino acids with the C-S-H surface. Negatively charged residues (Asp) interact electrostatically via Ca^{2+} bridged ions (left), amide groups (Asn) via both H-bonds and Ca^{2+} bridging due to a partial negative charge on the oxygen (middle) and hydrophobic residues (Leu) via Van der Waals interactions (right).

hydrogen bonding (especially amides for additional Ca^{2+} mediated adsorption) become favored for specific adsorption at pH values ≥ 12 , which are the conditions in real cementitious systems. These findings suggest a fundamentally different polymer design of a family of new strong C-S-H binding addi-

tives, which in turn will influence the physical properties of cement. Finding the right fractions of the various interactions will be a challenge for the future development of cement additives.

Experimental Section

pH Stability of Phage Display Library and Biopanning: A Ph.D.-12 phage peptide library (catalog no. E8110S) was purchased from NEB (New England Biolabs, MA) and used as received. 0.5 ml of TBS-buffer and different concentrated solutions of NaOH, respectively were prepared to give pHs of 7.6, 10, 11, 12, 12.5 and 13 after the addition of 5 μl of the original phage display library (10^{13} pfu/mL). The samples were gently rocked (500 rpm, 30 minutes). After addition of 0.5 mL TBS to partly neutralize the solutions, a dilution series with TBS was prepared and phage titrating with 10 μL of the obtained solutions was performed on LB medium/IPTG/X-gal plates.

The same Ph.D.-12 phage peptide library was used to perform biopanning against different C-S-H samples. An accurate description is provided in the SI.

C-S-H Synthesis: C-S-H suspensions were prepared by reacting lime and silica in appropriate ratios in a stirred aqueous suspension (pozzolanic method).^[39] The name of the samples refers to the lime to silica ratio (Ca/Si) of the mix. For details see SI.

Total Organic Carbon Analysis: Peptides were ordered and synthesized from Selleck Chemicals (Houston, TX) in desalted quality

Table 5. Adsorption of chosen identified sequences from the PD experiments and amino acid variations on C-S-H 1.0 and 1.5. Positively charged amino acids are highlighted in grey, negatively charged ones in black and H-donating groups in light grey. Arginine and Lysin are only partly protonated at the corresponding conditions and hence shaded. Glutamic acid in Var_5 was included as the equivalent motif with aspartic acid gave negative adsorption rates.

Name	Sequence	Adsorbed on C-S-H 1.0 [%]	Adsorbed on C-S-H 1.5 [%]
Pep E	L P N H L G A I L R A D	30.5	26.0
Var_4	R D R D R D R D R D R D	37.7	44.7
Var_5	R A E R A E R A E R A E	43.8	41.2
Var_6	A R A A D A A R A A D A	46.6	28.7
Var_7	D A D A D A D A D A D A	28.7	11.4
Var_8	L P N H L G A I L S A S	60.2	43.5
Var_9	S L L L S S S L L L S S	55.4	43.6
Var_10	S S S L L L L L L S S S	30.3	36.6
Var_11	Poly Lys (111 – 177)	78.9	67.1
Var_12	Poly Asp (18 – 26)	76.2	68.0

and like poly-DL-lysine (25000–40000 g/mol, Sigma Aldrich) and poly aspartic acid (Baypure DS100, 2000–3000 g/mol) used as received without further purification. C-S-H suspensions were synthesized via the pozzolanic method. For this, CaCO₃ was heated to 1000 °C for 4.5 hours to give CaO and used within 1 h in order to avoid carbonation. Silica Aerosil was dissolved in decarbonated degased MilliQ water and peptide stock solution was added to give final peptide concentrations of 1.2 g/L (C/S = 1.0 and 1.5) and 2.0 g/l (C/S = 0.66), respectively. Then, CaO was added in the proper amounts to give total volumes of 10 mL C-S-H suspension with a liquid to solid ratio (l/s) of 50 and calcium to silicon ratios (Ca/Si) of 0.66, 1.0 and 1.5. The suspensions were stirred under argon atmosphere and room temperature for 3 weeks. After filtration (200 nm pore size), the filtrate was prepared for TOC measurements. For this purpose, 1 mL of filtrate was diluted with 18.6 ml H₂O in MilliQ quality and acidified with 0.4 ml concentrated H₃PO₄. To obtain the exact TOC values for the peptide stocks, additional reference samples were prepared with these solutions. The amount of remaining peptide in solution can then be calculated and correlated with the theoretically obtained amount with the difference being adsorbed on C-S-H. The TOC analyzer utilized in this study is a Shimadzu TOC-VCN + TNM-1.

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