The Influence of Cytochrome C on the Polycondensation of Silicic Acid

By D. Gebauer and J. Woenckhaus*

Institut für Physikalische Chemie und Elektrochemie, Lehrgebiet A,
Universität Hannover, Callinstr. 3 A, D-30167 Hannover, Germany

Silicic Acid / Polycondensation Kinetics / Cytochrome C / Biomineralization / Cryoscopy / Sterical Factor

The polycondensation kinetics of silicic acid was measured cryoscopically in a temperature interval between 5 °C and 35 °C. Unlike polyamines such as polyallylamine, polylysine or polyarginine, catalitical amounts of Cytochrom c have a distinct retarding influence on the polycondensation kinetics. The Arrhenius analysis of the experimental reaction rates with and without Cytochrome c determines an invariant activation energy of $E_A^{CC/wo} = (69.9 \pm 0.7) \text{ kJ/mol}$, while the pre-exponential factors differ by $\Delta\kappa_0 = (1 \times 10^3 \pm 8 \times 10^7) \text{ s}^{-1}$. The latter can be attributed to a three-times peioration of a sterical factor, which then indicates, that the adsorption of reactive silicic acid oligomers on the protein is dominating the reaction rate.

1. Introduction

The properties of silicates are of special interest because of their importance in material science. There has been a lot of research effort focussing on studies of silicate formation. A review of these studies is given in Ref. [1]. Complex and delicate silicate structures are also important in a number of living organisms [2]. In this regard *in vitro* synthesis of such structures by means of controlled silicate formation is the final purpose of biomineralization studies. However, the understanding of biomineralization is at its very early stage [3]. Even though of the recent progress, there is a general lack in basical research devoted to studies on reaction dynamics of the polycondensation of silicic acid and the influence of foreign species on these dynamics. Some earlier studies provide activation parameters and rate constants for the polycondensation under different conditions [4–13].

* Corresponding author. E-mail: juergen.woenckhaus@pci.uni-hannover.de

However, a serious problem concerning accurate chemical kinetics is, that even the initial polycondensation exhibits concurrent processes [1]. It is widely accepted that the polycondensation rate is dominated by the condensation at ionized silicic acid species, because the presence of a nucleophilic oxygen in charged species accelerates the reaction. Since the pK of silicic acid species decreases from monosilicic to oligosilicic acids, the early stages of the reaction are already affected by condensation on oligosilicic acids [14]. The pK of monosilicic acid is around 9.8 and only at $\text{pH} \approx 2 \dots 6$ the polycondensation is slow enough for the initial steps to be studied in detail.

The investigation of the first polycondensation stages and accurate knowledge of the dissolved species is the basis of the crystallization control. In addition to NMR studies such as [14], *e.g.*, mass spectrometric studies [15–17] can help to illuminate the condensation process. However, fundamental studies such as the characterization of activation parameters, which help to understand the purposive silicate formation, pave the way to manipulation of reaction dynamics. Recent studies, [3, 18] (references therein), show, that the silicic acid polycondensation process is activated by polyamines such as polyallylamine, polylysine and polyarginine. It is proposed, that these polyelectrolytes adsorb monomers and small oligomers onto amino groups bringing them together and favouring condensation. Thus, this activation is not a catalytic effect, *i.e.* there is no depression of activation energy barrier. However, catalytic effects of biosilification proteins like Silicatein on the polycondensation of silicic acid are postulated. In the view of purposive silicate formation, it might be interesting, how to slow down rather than speed up the reaction rates. If monomers and oligomers get adsorbed onto polyelectrolytes and if the adsorption onto a charged function is sufficiently stable, a spacial separation of adsorbed silicic acid species should slow down the reaction rates. This pre-requisite is valid for most proteins, as charged amino acids are arranged all over the protein surface while already small amounts of protein provide a huge surface in the reaction batch.

We investigated the influence of catalytical amounts of Cytochrome *c*, a protein of the mitochondrial respiratory chain, on the polycondensation kinetics in a biologically relevant temperature interval from 5 °C to 35 °C. Cytochrome *c* was chosen to exemplify a well studied protein, even so Cytochrome *c* is not a protein like Silicatein [19] involved in the silification process in organisms. As accurate chemical kinetics are only accessible under simplified assumptions, we characterized the polycondensation kinetics by the increase of the medial molecular weight. An Arrhenius analysis of this phenomenological polycondensation rates provides effective activation energies and pre-exponential factors. These parameters elucidate whether catalytical or sterical aspects in the interactions between the protein and silicic acid species dominate the polycondensation dynamics.

Fig. 1. Schematic setup of the cryoscopy apparatus. A: additional cooling unit, T: Julabo thermostat F 12 ME, St: stirrer, PC: computer, S: reaction mixture, C: reaction container, G: Krick/Merz MPMI 1004/300 Pt-resistance sensor gauge, Th: Anton Paar MKT 100 precision thermometer, USV: independent voltage feed-in. XXXX indicates displays. Solid arrows indicate the flow of thermostated liquid, dotted arrows indicate the flow of the tempering liquid to the additional cooling unit. Bold lines indicate electric connections, dashed lines indicate a thermal isolation of the environment.

2. Experimental

We utilized a precision cryoscopy apparatus set up as shown schematically in Fig. 1. It consists of a computer controlled Julabo¹ F 12 ME thermostat coupled with an additional, self-constructed cooling unit to provide an improved cooling power of 2.2 kW at 20 °C. The heating power is 2 kW. The thermostat adjusts the temperature of the tempering liquid flowing through a double wall container (V2A) that acts as a reaction-cell. For the purpose of homogenisation, the solution is permanently stirred. The container is capped by a rubber-nyliner allowing a Pt-resistance-sensor-gauge Krick/Merz MPMI 1004/300 to protrude into the solution. The resistance of the sensor gauge is determined by a resistance measurement chain using an Anton Paar² MKT 100 precision thermometer. Since line voltage fluctuations compromise the measurement, the precision thermometer is decoupled from the line voltage using an independent voltage feed-in (USV). The entire measurement chain (MPMI 1004/300 and MKT 100) was calibrated at the calibration laboratory Klasmeier Kalibrier- und Meßtechnik GmbH in Fulda, Germany. The interpolation points of this three-point-calibration were the mercury melting point (−38.83440 °C, ITS-90, melting point cell), water triple point (+0.01000 °C,

¹ www.julabo.de

² www.Anton-Paar.com

ITS-90, triple point cell) and the gallium melting point (+29.76460 °C, ITS-90, melting point cell).

Before addition of tetramethylorthosilicate (TMOS), the freezing point of the pure solvent (bidistilled water, pH = 4.00 hydrochloric acid) was determined. The freezing point, which slightly depends on the barometric pressure, was around $-0.002\text{ }^{\circ}\text{C}$ in all experiments. The variation of the freezing point as a function of the pressure ΔT_p is calculated from the freezing point at standard conditions $T_m^{\oplus}$, the change in pressure Δp , the change in volume during freezing ΔV and the standard melting enthalpie $H_m^{\oplus}$ to $\Delta T_p = \frac{T_m^{\oplus} \Delta p \Delta V}{H_m^{\oplus}}$. Here, ΔV can easily be obtained from the specific volumes of water and ice at $0\text{ }^{\circ}\text{C}$. Barometric pressure usually fluctuates within 50 mbar. The resulting change of the freezing point of approximately 0.4 mK is small enough to be neglected. Furthermore, such barometric pressure fluctuations rarely occur during the measurement duration of approximately 12 h.

After the solvent heating to $40\text{ }^{\circ}\text{C}$ TMOS was added, and TMOS prehydrolysis was performed for about 7 min. Then the reaction was cooled down at a rate of approximately 2 K per min and the freezing point was determined. In the experiments with Cytochrome c, an aliquot Cytochrome c solution (in bidistilled water, pH = 4.00, hydrochloric acid) was added to the still frozen prehydrolysis. The medial silicic acid concentration after prehydrolysis was 0.115 mol/L in all experiments. The concentration of Cytochrome c was 1×10^{-5} mol/L in all experiments and caused no observable depression of freezing point in our experimental setup, as the depression of freezing point for this concentration is 18.5 μK . Cytochrome c from horse heart (*equus caballus*, Sigma-Aldrich, product number C-7752) was used in all experiments. The pH of the reaction batches was monitored to be constant in the studied temperature interval.

The solutions were heated to the reaction temperature at an initial rate of approximately 3 K per min ($5\text{ }^{\circ}\text{C}$ to $35\text{ }^{\circ}\text{C}$) then and the freezing point was determined in the interval of one hour. The cryoscopic progression is shown in Figs. 2 and 3. The depression of freezing point is calculated from the difference between the freezing point of the pure solvent and the freezing point after a certain reaction time. Fig. 3 shows the temperature curve and reaction time. The time for temperatures below $0\text{ }^{\circ}\text{C}$ can be neglected, since the kinetics become very slow at this low temperatures. However, the time during the heating and cooling periods cannot be neglected. The interval between two measurements of the freezing point can be calculated as the time period between passing $0\text{ }^{\circ}\text{C}$ and the end of the programmed reaction temperature: the initial heating is faster than cooling because of the temperature dependence of the cooling efficiency, which is inversely proportional to the temperature difference. When approaching the reaction temperature, heating of the reaction mixture slows down, because the thermostat adjusts the heating power and thus reduces the temperature gradient between tempering liquid and reaction mixture. Effectively, the integrals of heating and cooling phase are approximately

Fig. 2. Determination of the freezing point: Raw data are shown. The freezing point T_m is determined as shown. The lower plot shows the enlarged area indicated by dashed lines in the upper plot.

the same (see Fig. 3). Hence, one can assume that the full reaction temperature is effective for the predefined heating period. The period is 56 min in all experiments. The maximum deviation observed for one heating cycle is 1.5 min. In the error analysis the approximations were accounted for with an assumed total uncertainty of 5 min.

3. Results and discussion

The progression of the medial molecular weight $M_{\text{rel.}} = \frac{M_{\text{silicic acid}}}{M_{\text{measured}}}$ displays a temperature dependency and a distinct influence of Cytochrome c on the polycondensation rate (see Fig. 4, data see Tables 1 and 2). The linear increase of the

Fig. 3. Temperature curve and reaction time: Raw data are shown. The effective reaction time was assumed as the difference between two dotted lines a and b, the simplifications made are discussed in the text. The grey shaded areas give the integrals referred to in the text.

relative molecular weight can be represented to good approximation as a function of rate κ and time t by

$$M_{\text{rel.}} = 1 + \kappa t \quad (1)$$

until a relative molecular weight of approximately 4.5 is reached. Eq. (1) is a phenomenological derivation based on the obtained data as shown in Fig. 4. The regression provides the polycondensation rates κ_{CC} in presence and κ_{wo} in absence of Cytochrome c. The values are shown in Table 3. The temperature dependency of the polycondensation rates can be described by an Arrhenius approach

$$\kappa(T) = \kappa_0 e^{-\frac{E_A}{RT}}.$$

The Arrhenius plot is shown in Fig. 5. The values of $T = 5^\circ\text{C}$ ($\approx 0.0036 \text{ K}^{-1}$) can be neglected, as the reaction is very slow and the polycondensation rate can hardly be determined.

Already visual inspection of the data indicates a parallel slope. Indeed, a linear regression with identical slopes for both plots is maintain-

Fig. 4. Overview of the increase of relative molecular weight M_{rel} in presence (lower) and absence (upper) of Cytochrome c. All curves average four measurements. Only one exemplary error indicator is shown for clarity, indicating the 1σ -standard deviation of the individual measurements.

able to experimental accuracy. The fit provides $\kappa_0^{\text{wo}} = (2 \times 10^8 \pm 6 \times 10^7) \text{ s}^{-1}$ and $\kappa_0^{\text{cc}} = (7 \times 10^7 \pm 2 \times 10^7) \text{ s}^{-1}$ and a common activation energy $E_{\text{A}}^{\text{CC/wo}} = (69.9 \pm 0.7) \text{ kJ/mol}$. The difference of the pre-exponential factor is $\Delta\kappa_0 = (1 \times 10^8 \pm 8 \times 10^7) \text{ s}^{-1}$. This difference can be interpreted as a change in a sterical factor F ,

$$\frac{\kappa_{\text{wo}}}{\kappa_{\text{cc}}} = \frac{\kappa_0^{\text{wo}} e^{-\frac{E_{\text{A}}^{\text{CC/wo}}}{RT}}}{\kappa_0^{\text{cc}} e^{-\frac{E_{\text{A}}^{\text{CC/wo}}}{RT}}} = \frac{\kappa_0^{\text{wo}}}{\kappa_0^{\text{cc}}} = \frac{F_{\text{wo}}\kappa_0}{F_{\text{cc}}\kappa_0} = \frac{F_{\text{wo}}}{F_{\text{cc}}} \quad (2)$$

Table 1. Obtained data in absence of Cytochrome c. σ denotes the $1-\sigma$ deviation of the individual measurements. The data were corrected to $M_{\text{rel.}}(t=0) = 1.000$ by parallel translation.

without t (min)	35 °C		30 °C		25 °C		20 °C		15 °C		10 °C		5 °C	
	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ
0	1.0000	0.0525	1.0000	0.0495	1.0000	0.0910	1.0000	0.0655	1.0000	0.0710	1.0000	0.0147	1.0000	0.0241
56	1.8093	0.3731	1.4372	0.2107	1.2775	0.1694	1.1601	0.1497	1.1120	0.0505	1.0511	0.0613	1.0305	0.0496
112	2.7065	0.6711	2.0078	0.4352	1.5735	0.3628	1.3845	0.2860	1.1983	0.0864	1.0854	0.0427	1.0137	0.0422
168	3.7079	1.0710	2.5091	0.6749	1.8330	0.3770	1.5782	0.3781	1.3444	0.1737	1.1334	0.0782	1.0572	0.0547
224	4.5734	1.9336	3.1674	1.2614	2.3287	0.7345	1.8735	0.5951	1.4400	0.1679	1.1783	0.0787	1.1234	0.0840
280	4.5887	1.4762	3.4401	0.9736	2.6367	0.8263	2.1802	0.8714	1.6316	0.2791	1.2261	0.1378	1.0835	0.0861
336	5.7584	2.4023	4.2609	1.8123	2.9236	0.9088	2.2806	0.6854	1.7558	0.3109	1.2715	0.1771	1.1630	0.1065
392	5.6797	1.5841	4.4963	1.2391	3.3353	0.6849	2.3955	0.6887	1.8921	0.3734	1.2931	0.1947	1.1777	0.1024
448	6.7388	3.4739	4.6106	1.1169	3.5226	0.7412	2.8162	0.7242	2.0307	0.4202	1.3468	0.2592	1.1873	0.1402
504	5.5011	1.2459	4.9078	0.9927	3.9114	0.4685	2.9987	0.8213	2.2213	0.4847	1.5480	0.2650	1.3169	0.1998
560	7.0494	2.2226	5.6568	1.1561	4.0060	0.8187	3.4128	0.8976	2.4063	0.5436	1.6885	0.4086		
616	6.6462	2.4007	5.0252	1.0803			3.2768	0.8886	2.6247	0.7375	1.8189	0.4099		
672	6.6171	2.6365					3.6821	0.8174	2.7702	0.7535	1.8455	0.5056		
728									2.7631	0.6620	1.9753	0.5547		
784											2.1527	0.6161		
840											2.2415	0.7419		
896											2.2358	0.5414		

Table 2. Obtained data in presence of 1×10^{-5} mol/L Cytochrome c. σ denotes the 1- σ deviation of the individual measurements. The data were corrected to $M_{\text{rel}}(t = 0) = 1.000$ by parallel translation.

with	35 °C		30 °C		25 °C		20 °C		15 °C		10 °C		5 °C	
<i>t</i> (min)	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ	$M_{\text{rel.}}$	σ
0	1.0000	0.0352	1.0000	0.0437	1.0000	0.0147	1.0000	0.0499	1.0000	0.0079	1.0000	0.0319	1.0000	0.0285
56	1.2764	0.0648	1.1252	0.0670	1.0569	0.0623	1.0480	0.0507	1.0266	0.0462	0.9816	0.0411	0.9893	0.0614
112	1.4710	0.1217	1.3258	0.0699	1.1435	0.0527	1.1169	0.1042	1.0619	0.0595	0.9710	0.0196	0.9776	0.0493
168	1.8627	0.1661	1.4854	0.1075	1.2265	0.0594	1.1360	0.0710	1.1044	0.0352	1.0393	0.1009	0.9800	0.0667
224	2.1814	0.3444	1.7079	0.1413	1.4584	0.1477	1.2464	0.0914	1.0663	0.0945	1.0246	0.0495	1.0219	0.0566
280	2.2224	0.2554	1.9742	0.2137	1.5473	0.0611	1.3307	0.1152	1.1183	0.0918	1.0566	0.0245	1.0198	0.0271
336	3.0969	0.7888	2.0288	0.1995	1.6116	0.1487	1.3656	0.1094	1.1835	0.1018	1.0812	0.0515	1.0067	0.0191
392	3.3499	0.3667	2.2395	0.2220	1.6771	0.0908	1.5544	0.1396	1.1573	0.0439	1.1215	0.0549	1.0588	0.0313
448	3.7495	0.6794	2.6343	0.4610	1.8160	0.0721	1.6869	0.1640	1.2927	0.1248	1.1368	0.0779	1.0938	0.1006
504	3.8648	0.6463	2.6534	0.4555	1.9799	0.3068	1.7440	0.1883	1.3686	0.1347	1.1670	0.1017	1.0562	0.0363
560	3.8813	0.5717	2.8749	0.3936	2.3318	0.3667	1.7938	0.1503	1.4048	0.0711	1.1912	0.0679	1.0797	0.0038
616	4.9322	1.0379	3.1634	0.6115	2.5641	0.2534	1.9283	0.1054	1.5153	0.1809	1.3004	0.0925		
672	4.3553	1.0145	3.8152	0.7417	2.5253	0.0837	1.9722	0.1701	1.6084	0.1240	1.2904	0.0866		
728	4.9502	1.0347	3.4602	0.5038	2.4639	0.2414			1.5282	0.0661	1.2937	0.0979		
784									1.6124	0.1532				

Table 3. Polycondensation rates determined by linear regression (Eq. (1)) of the experimental data shown in Fig. 4. κ_{wo} indicates the rates without and κ_{CC} indicates the rates with Cytochrome c, respectively.

T (°C)	κ_{CC} (s ⁻¹)	κ_{wo} (s ⁻¹)
35	9.40×10^{-5}	2.69×10^{-4}
30	6.20×10^{-5}	1.59×10^{-4}
25	3.87×10^{-5}	9.86×10^{-5}
20	2.20×10^{-5}	6.73×10^{-5}
15	1.41×10^{-5}	4.36×10^{-5}
10	8.00×10^{-6}	2.51×10^{-5}
5	3.13×10^{-6}	2.11×10^{-5}

Fig. 5. Arrhenius diagram in a temperature range from 5 °C to 35 °C. ‘A’ depicts data and regression of the data in absence of Cytochrome c, ‘B’ in presence of Cytochrome c, respectively. The data indicate invariant slopes, *i.e.* the activation energy stays unaffected. See text for detailed discussion.

Eq. (2) provides the ratio of sterical factors without F_{wo} and with F_{CC} Cytochrome c by the ratio of the pre-exponential factors κ_0^{wo} and κ_0^{CC} , respectively, to $\frac{\kappa_0^{wo}}{\kappa_0^{CC}} = \frac{F_{wo}}{F_{CC}} \simeq 3$. Thus, the sterical factor gets three times more unpropitious in presence of Cytochrome c. As the activation energies are invariant for the presence and absence of Cytochrome c, there is no catalytic influence of Cytochrome c on the polycondensation of silicic acid. The influence on the sterical factor can easily be rationalized by an adsorption of reactive oligomers. Undoubtedly, it is not the silicic acid monomer getting adsorbed, as this would

have to be indicated by an accelerated increase of M_{rel} in the reaction mixture. An insignificant amount of adsorbed monomers cannot explain the retarding effect either. If oligomers get adsorbed, the reactive surface of the oligomer is obstructed by the protein. The amount of adsorped oligomers might be quite small, if only few oligomers exist that explicitly dominate the reaction rate. Thus, it is evident, that the polycondensation in presence of Cytochrome c is retarded because of the adsorption of reactive oligomer species onto the protein surface. Which species get adsorbed could yet not be clarified by means of ESI-MS and NMR.

Acknowledgement

We acknowledge financial support by the Deutsche Forschungsgemeinschaft (WO656).

References

1. R. K. Iler, *The Chemistry Of Silica*. John Wiley & Sons, New York (1979).
2. S. Mann, *Biominalisation – Principles and Concepts in Bioinorganic Materials Chemistry*. Oxford University Press, Oxford (2001).
3. S. V. Patwardhan, S. J. Clarson, and C. C. Perry, *Chem. Commun.* (2005) 1113.
4. G. A. Icopini, S. L. Brantley, and P. J. Heaney, *Geochim. Cosmochim. Acta* **69**(2) (2005) 293.
5. W. Flemming, *Z. Phys. Chem.* **41** (1902) 427.
6. C. B. Hurd, *Chem. Rev.* **22** (1938) 403.
7. C. B. Hurd and R. W. Barclay, *J. Phys. Chem.* **44** (1940) 847.
8. C. B. Hurd and A. J. Marotta, *J. Am. Chem. Soc.* **62** (1940) 2767.
9. C. B. Hurd *et al.*, *J. Am. Chem. Soc.* **66** (1944) 388.
10. S. S. Penner, *J. Polym. Sci.* **1** (1946) 441.
11. A. P. Brady, A. G. Brown, and H. Huff, *J. Colloid Sci.* **8** (1953) 264.
12. G. B. Alexander, *J. Chem. Soc.* **76** (1954) 2094.
13. A. D. Bishop, Jr. and J. L. Bear, *Thermochim. Acta* **3** (1972) 399.
14. D. Hoebbel, W. Wieker *et al.*, *Z. Anorg. Allgem. Chem.* **400** (1973) 148; **405** (1974) 163; **428** (1977) 43; *J. Chromatogr.* **119** (1976) 173.
15. P. Bussian, F. Sobott, B. Brutschy, W. Schrader, and F. Schüth, *Angew. Chem.* **112**(21) (2000) 4065.
16. K. Eggers, T. Eichner, and J. Woenckhaus, *Int. J. Mass Spec.* **244** (2005) 72.
17. S. Zink, T. Eichner, M. Schnell, and J. Woenckhaus, *Z. Phys. Chem.* **219** (2005) 1355.
18. T. Coradin and P. J. Lopez, *ChemBioChem.* **3** (2003) 1.
19. J. N. Cha *et al.*, *Proc. Natl. Acad. Sci. USA* **96** (1999) 361.