ELSEVIER

Journal of Crystal Growth 205 (1999) 375–381

JOURNAL OF CRYSTAL GROWTH

www.elsevier.nl/locate/jcrysgro

Physical and chemical properties of gels Application to protein nucleation control in the gel acupuncture technique

Abel Moreno[a,*], Gabriela Juárez-Martínez[a], Tomás Hernández-Pérez[b], Nikola Batina[c], Manuel Mundo[d], Alexander McPherson[e]

[a]*Departamento de Bioquímica, Instituto de Química, U.N.A.M. Circuito Exterior, C.U. México, D.F. 04510, Mexico*
[b]*Departamento de Ciencias Básicas, Universidad Autónoma Metropolitana, Azcapotzalco, Mexico*
[c]*Departamento de Química, Universidad Autónoma, Metropolitana-Iztapalapa, Mexico*
[d]*Department of Nematology, University of California, Riverside, USA*
[e]*Department of Biochemistry and Molecular Biology, University of California, Irvine, USA*

Received 1 May 1998; accepted 24 April 1999
Communicated by N.E. Chayen

Abstract

In this work, we present a new approach using analytical and optical techniques in order to determine the physical and chemical properties of silica gel, as well as the measurement of the pore size in the network of the gel by scanning electron microscopy. The gel acupuncture technique developed by García-Ruiz et al. (Mater. Res. Bull 28 (1993) 541) García-Ruiz and Moreno (Acta Crystallogr. D 50 (1994) 484) was used throughout the history of crystal growth. Several experiments were done in order to evaluate the nucleation control of model proteins (thaumatin I from *Thaumatococcus daniellii*, lysozyme from *hen egg white* and catalase from *bovine liver*) by the porous network of the gel. Finally, it is shown how the number and the size of the crystals obtained inside X-ray capillaries is controlled by the size of the porous structure of the gel. © 1999 Elsevier Science B.V. All rights reserved.

PACS: 36.20; 87.15; 81.10

Keywords: Protein crystal growth; Silica gels; Nucleation control; Lysozyme; Thaumatin I; Gel acupuncture technique

1. Introduction

Nowadays, the availability of high-quality single crystals for 3D X-ray characterisation is crucial in several fields of materials science. In the case of biological macromolecules, most of the investigations have been focused on determining the best crystallisation conditions for a specific system. Although there have been some approaches to overcome this handicap, the next difficult part of the research needs to be focused on choosing the best crystal growth technique in order to obtain huge

* Corresponding author. Tel.: + 52-5-6224568; fax: + 52-5-6162217.
E-mail address: carcamo@servidor.unam.mx (A. Moreno)

0022-0248/99/$ - see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0022-0248(99)00263-8

single crystals. One interesting approach for obtaining high-quality single crystals is to reduce or at least minimise the convection phenomena. There are three possible ways for doing this. The first is to grow crystals in low-gravity conditions. The second as shown by García-Ruiz and Moreno [1,2], is to grow crystals inside thin capillary tubes just by keeping the unidirectional transport of the molecules through the true protein solution. The third is to grow crystals inside the porous network though, in the case of macromolecules, this seems a non-recommended crystal growth method. The knowledge about the physical and chemical properties of gels is important for the gel acupuncture technique. This has been an alternative technique for growing crystals of biological macromolecules. For this particular case, the gel works as a porous network that permits the transportation of the ions and keeps the capillaries well fixed in vertical position. These are the reasons for studying silica gels. The aim of this research will be focused on determining these physical and chemical properties of the silica gels obtained for chemical synthesis as a polymeric reaction. There are some reports about the global porosity of gels and their fractal structure derived from tetraethoxysilane (TEOS), where microtexture has been studied [3]. Differential scanning calorimetry has been used to measure the pore size distribution (PSDs) from the melting and freezing curves of water confined in pores. Thermal porosimetry has been established by combining some physical properties applied to 12 kinds of commercial silica gels [4,5]. The determination of pore size distribution from sorption isotherms applying the percolation theory has been published by Zhdanov et al. [6]. Silica membranes, their preparation and the structure of microporous, have been published in Ref. [7]. The pore surface, characteristics of macroporous silica-gels prepared from polymer containing solution, has been published by Nakanishi et al. [8]. There are many reports about the use of gels as crystal growth cells that take into account their advantages for keeping the diffusional path all over the experiment. Revision and data to prepare silica gels from different ways have been published by Roberts et al. [9]. Many of the problems, found in the crystal growth of proteins, could be overcome if the methodology for preparing the gels is well established and all the parameters that control the gelling phenomena are well understood.

2. Chemicals

Thaumatin I (Sigma, T-7638), Lysozyme (Sigma, L7773) and catalase (Sigma, C3155) were used as purchased without further purification. Sodium potassium tartrate, sodium chloride and ammonium sulphate were used as precipitating agent solutions, respectively. All the solutions were prepared with double-distilled water. The layer that holds the capillaries was made of silica, by mixing appropriate volumes of sodium silicate solution (Aldrich, 33,844-3) with a specific gravity of 1.06 g/cm^3 and acetic acid 1 M. The gels obtained by hydrolysis of tetramethoxysilane (TMOS, FLUKA catalogue number 87682) were prepared by mixing the TMOS with double-distilled water, following the experimental set-up described in the paragraph below. For evaluating the nucleation phenomena (using the implementation of the gel acupuncture technique) the pH values of the silica gel were 8 and 10 for thaumatin and 4.0 and 6.0 for lysozyme. We used phosphate buffer (100 mM) to maintain the protein solution at pH 7.0 for thaumatin and Tris-HCl pH 9 (100 mM) for catalase. Sodium azide solution was added to the precipitating agent at 0.1% (w/v).

3. Experimental procedure

The gels used in this work were hydrogels. The gels are two-component media with water molecules soaking a porous flexible polymer network. The gelation process corresponds to the setting of a polymeric cluster stretching over the whole volume of solution. This process is either reversible for physical gels, like gelatine and agarose gels, which are obtained by decreasing the temperature, or irreversible for chemical gels (such as silica or polyacrilamide gels) which are obtained from the formation of strong bonds. Although universal “best” gel cannot be recommended [10], silica and agarose gels have proved their efficiency for growing macromolecular compounds [11,12].

3.1. Silica gels obtained by neutralisation of sodium metasilicate

The silica gels have been used for many years as crystal growth cells, because their porous network permits the diffusion of several ions and of some large polymers (like polyethyleneglycols and other macromolecules). These silica gels were basically obtained from the neutralisation of sodium metasilicate solution (1.06 g/ml) with acetic acid 1 M.

Sodium metasilicate solution 1.06 g/ml was prepared by the following equation:

$$V_{ss} = \frac{V_a(0.06)}{1.39 - \rho^{20^\circ C}}$$

where V_{SS} is the volume of sodium silicate solution to be taken from the bottle of the chemical (ALDRICH Cod. 33,844-3). V_a is the volume to be measured in a volumetric flask, 1.39 is the density of the sodium metasilicate from the chemical and $\rho^{20^\circ C}$ is the density of water at room temperature.

Once the solution of sodium metasilicate has been prepared, a titration plot is then obtained in order to have several gels at different pH values for determining the gelling time and the reproducibility in the experiments. This could be a crucial parameter in the application of these gels to protein crystallisation.

3.2. Silica gels from tetramethoxysilane (TMOS) solutions

These silica gels were prepared from siloxanes, tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS). Both siloxanes are liquids, soluble in alcohols (ethanol or methanol) but not directly in water. In this particular case, two steps of the gelling process have to be considered separately: hydrolysis and polymerisation. These gels from TMOS and TEOS, used for these investigations, were prepared following the procedure described by Roberts et al. [9].

4. Results and discussion

4.1. The physico-chemical properties of gels and the gelation time as a function of pH

The three-dimensional network of the solid polymeric phase forming the gel leaves a fractal distribution of porous filled by the liquid phase. Due to the small average pore size, the gel structure prevents convective mass flow while allowing the Brownian motion of ions and small clusters through the intraporous phase. The gelation time depends on many parameters: nature and concentration of species in solution, pH, temperature. In case of thermally stable solutions, one can shorten this time by increasing temperature. Practical considerations require gelation times to take place in less than a couple of days. One estimates that the gel is set when it resists pouring. It must stick to the crystal growth cells walls. It can look somewhat opalescent but without heterogeneities such as fissures.

There are some useful ways to determine the gelation time for many type of gels. The first method consists of measurements from the polymerisation process, the time when the gel structure is well fixed to the walls of the container. This means the time when the gel does not pour anymore. It is obvious that the whole amount of water is encapsulated inside the porous network. As a result of this macromolecular inter-crossing from the polymeric reaction, the structure of the network expels water drops by itself. The process of expelling drops of water is usually called syneresis, which is the second technique for estimating the gelation time. This is UV-VIS Spectroscopy using quartz cells and measuring at 250 nm. This gelation time as a function of the pH is shown in the Fig. 1 for silica gels obtained from the neutralisation of sodium metasilicate solution with acetic acid. From this plot, it is easy to see that the polymeric reaction works faster at a pH's close to neutralisation than those pH's far from this value. For this determination a VARIAN CARY E-1 Spectrophotometer was used for the measurements. The estimated gelation time was that where the absorbency was constant.

4.2. Pore size determination of the network of polysiloxanes by scanning electron microscopy

The main purpose of this part of the investigation is focused on determining the size of porous network in order to study the transport phenomena of several chemicals useful in protein crystallisation. It is important to mention that most of the

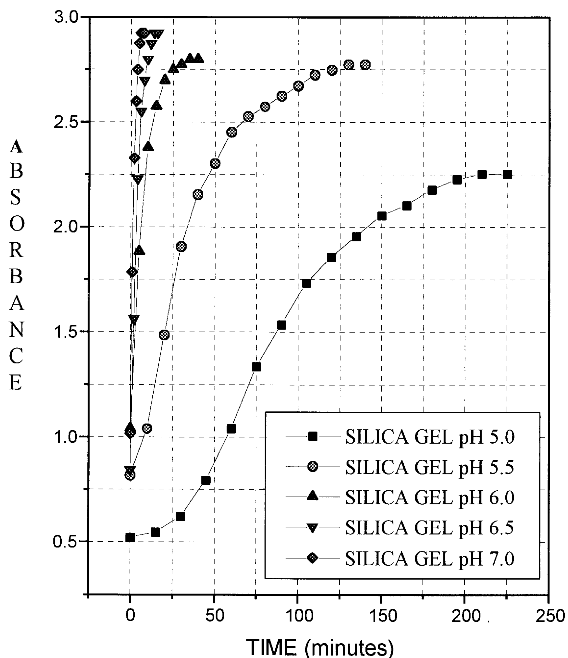


Fig. 1. Plot of gelation time for silica gels at different pH values. All data were collected at 250 nm.

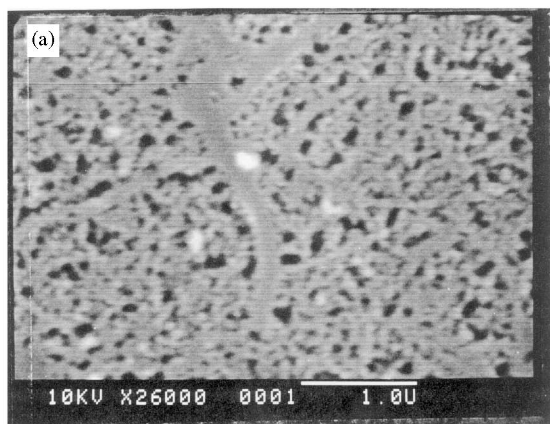
approaches had been done based on the idea that porous size is nearly the same in silica obtained by titration of sodium metasilicate with acetic acid. In the case of the gels obtained by hydrolysis of tetramethoxysilane (called TMOS), the size of the porous network depends on the concentration of TMOS. To know this is crucial in order to design a new experimental crystallisation protocol. Both transport and crystal growth phenomena depend on porous size distribution. This plays an important role in controlling the velocity of ions and the number of nuclei obtained all over the crystallisation phenomena. Therefore, the aim of this research was focused on estimating the pore size distribution (PSDs) and the evaluation of the pore size by scanning electron microscopy. For this investigation, a scanning electron microscope JEOL JSM-35 was used for the analysis of the samples.

Sample preparation: 1.4 ml of sodium metasilicate solution (1.06 g/ml) were added drop wise to 1.0 ml of acetic acid 1 M. This solution was added to a cylinder reservoir fixed with vacuum grease over a glass cover slide (12 mm in diameter). Once the gel was obtained ethanol solution was added onto the surface of the gel to remove water from the network. This procedure was repeated every 60 min 8-fold. After this removal the gel was put within a disposable sterile suspension culture dishes (Corning 25050-35), and dehydrated ethyl alcohol was added onto the surface of the gel to permit the final removal of water. After 24 h the alcohol was removed and the gels were dried at critic point to avoid the structure damage to be analysed in scanning electron microscopy. The critic point drying is a useful technique to remove ethanol molecules and replace these for CO_2 (0–5°C). This removal of ethanol was carried out as follows: once the water was removed by using absolute ethanol, the samples were introduced to the critic point drying equipment. Liquid CO_2 was then added to replace the EtOH molecules by CO_2 molecules. This exchange between EtOH and CO_2 was done twice every 20 min. This drying is necessary to keep the structure of the gel without changes in the SEM analysis.[1]

The silica gels, obtained by neutralisation of sodium metasilicate solution (specific gravity 1.06 g/ml) with acetic acid 1 M, showed random pore sizes distribution ranging from 50 to 150 nm. These results are shown in Fig. 2a for pH 6 and Fig. 2b for pH 10. From this, it is possible to say that the gels at alkaline pH had a more homogeneous pore size distribution, whereas the gels obtained at acidic pH show shorter size and more random pore size distribution than those prepared at pH 10.

The same methodology has been applied in order to study the porous network of gels obtained by hydrolysis of tetramethoxysilane at four different concentrations (2, 5, 10 and 20% v/v). This gel had different pore size distribution and this depends on the concentration of the solution. But neither the porous network has been systematically determined nor has the transport phenomena in the gel acupuncture technique yet been studied or

[1] This part of the research was done in the Department of Nematology, University of California, Riverside, USA.



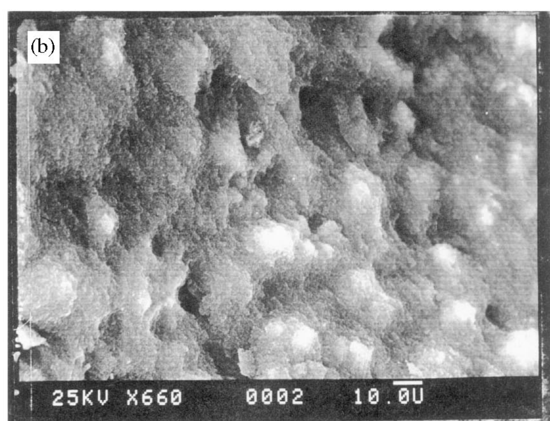


Fig. 2. SEM micrographs of gels from sodium metasilicate solutions. They correspond to silica gel: (a) pH 6.0 and (b) pH 10.

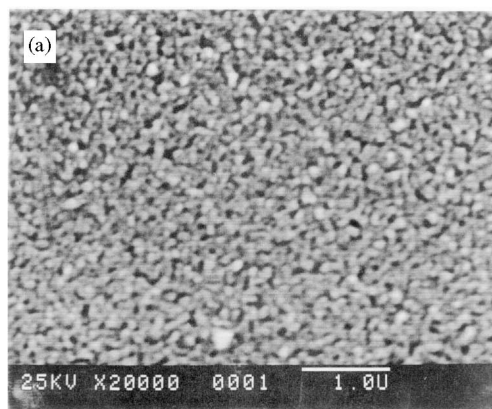


Fig. 3. SEM micrographs of gels from tetramethoxysilane solutions. The porous network was prepared at different concentrations of TMOS: (a) 20% (v/v) and (b) 10% (v/v).

published. From the microphotographs obtained by SEM, the TMOS gels at 10% (v/v) showed bigger pore size (Fig. 3a) than those synthesised at 20% (v/v) of tetramethoxysilane (TMOS), this is shown in Fig. 3b. The estimated average pore size at 10% (v/v) of TMOS was ranging from 150 to 250 nm and from 50 to 100 nm for the case of TMOS gels at 20% (v/v). It is important to note that the structure of the gels of TMOS at concentrations below 5% (v/v) decayed when the vacuum of the SEM measurements were done.

4.3. Solubility of silica gels

The solubility of silica gels as a function of pH has been described by Henisch [10]. In this aforementioned work, the gelling process was evaluated and the time for a quick gelation at pH ranging from pH 7.5 to 8.5 at 17.5°C was found to be around 100 sec. The trend of silica to dissolve increased at pH below pH 3 and above pH 10.5 as has been published by Iler [13]. From our experimental results, we noticed that the gels obtained at alkaline pH from sodium metasilicate solution were compressed once the ethanol molecules replaced the water molecules in the polymeric network. The original size was reduced by up to 35% due to this molecular exchange. Future investigations will be focused on determining this observation and a molecular simulation will be obtained based on porous network determination by AFM. On the other hand, gels obtained by the hydrolysis of

tetramethoxysilane did not show this structural compression when water molecules were replaced by ethanol in the preparation of samples for scanning electron microscopy.

5. Applications of silica gels

5.1. Application of silica gels to control the nucleation in the gel acupuncture technique

The glass cassettes used for testing the nucleation phenomena inside the capillary tubes by monitoring the influence of the penetration length, were prepared using the experimental set-up of the gel acupuncture technique [1,2]. This glass cassette consists of two 70×100 mm rectangular glass plates held apart by an U-shaped spacer covering the two longest and one of the shortest sides of the plates. After fastening the plates with clamps, the gap between them was approximately 4 mm. This glass vessel was then filled to a height of 30 mm with 2 cm^3 of a silica sol. Once gelled, the capillaries containing the protein solution are punctuated into the gel with a penetration length of 7 mm. Finally, 2 cm^3 of the precipitating solution is poured onto the gel. The uncovered shorter edge of the cassette is then closed with adhesive tape and the cassette is placed at room temperature (18 ± 1°C). Cylindrical capillaries with an internal diameter of 0.5 and 1.2 mm were used. Prior to the experiment, the capillaries were washed with double distilled water and acetone and dried air.

There was a remarkable influence of the porous network of the gel in controlling the size and number of the protein crystals. In the case of lysozyme grown in two silica gels prepared at two different pH's 4 and 6, the number of crystals was higher at pH 4 than that obtained at pH 6, which has the smaller radius of the pores of the web. The same nucleation behaviour was obtained in the case of crystallisation of thaumatin I and catalase. These results are shown in Fig. 4 for thaumatin grown in two different gels. For all cases the induction time

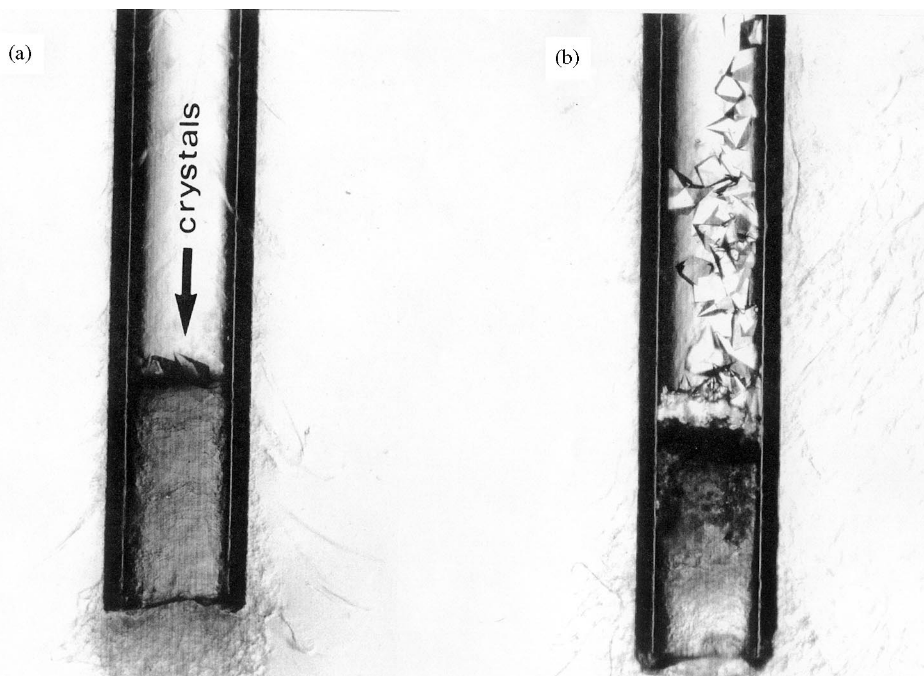


Fig. 4. Crystals of thaumatin grown in capillary tubes. The internal diameter of the capillary tube was 1.2 mm. The photographs correspond to crystals obtained in gels from sodium metasilicate solutions prepared at (a) pH 8 and (b) at pH 10. The arrow shows the zone where two single crystals were obtained in the gel with a closer pore size distribution of the silica gel (a).

for protein nucleation was larger in the compacted gel (close to pH of neutralisation), 6 for lysozyme and 8 for thaumatin and catalase. In addition, we estimated the influence of different penetration lengths upon nucleation and size of the crystals. In general terms, it is possible to say then that a larger penetration length (at constant pH of the gel) permits to control the nuclei number and the induction time for nucleation.

6. Concluding remarks

The silica gels obtained from the neutralisation of the sodium metasilicate solution (1.06 g/ml) with acetic acid show a random distribution of the pores when the solution is close to the pH of neutralisation. This trend has been shown from the current results and the average size is ranging from 50 to 150 nm for gels prepared at pH 6. On the other hand, far from this mentioned pH, e.g. pH 10, it is possible to say that the pores are bigger than those pores of the gel at pH 6 and showed more homogeneous average distribution throughout the polymeric network. For gels obtained by hydrolysis of tetramethoxysilane, higher concentration of this permit to obtain the lower pore size distribution. In this particular case (20% (v/v)) the average pore size was ranging from 50 to 100 nm. Lower concentration of TMOS (10% (v/v)) used to prepare gels made possible to obtain a higher average pore size of the polymeric web ranging from 150 to 250 nm. Finally, it is possible to control the nucleation phenomena in protein crystallisation by using closer pore size in the experimental set-up of the gel acupuncture technique, and as a consequence of the random pore size distribution, the diffusivity of ions is increased. The trend was observed for the case of thaumatin, lysozyme and catalase grown in capillary tubes using silica gels as a membrane dialysis in the experimental set-up of the gel acupuncture technique. Additionally, the nucleation phenomena are also controlled by the penetration length of the capillaries punctuated inside the gel surface used as a dialysis membrane, which permitted to monitor the transport of the precipitating agent for protein crystallisation experiments.

Acknowledgements

One of the author (A.M.) acknowledges grants from American Chemical Society and National Science Foundation (USA) as a visiting researcher at the department of Biochemistry, University of California, Riverside (USA). A.M. also acknowledges to Prof. Alexander McPherson, from the University of California now at Irvine, for giving all facilities to do this research. Finally, one part of this investigation was supported by CONACYT project number J27494-E. This is the contribution number 1675 from the Institute of Chemistry, UNAM.

References

[1] J.M. García-Ruiz, A. Moreno, C. Viedma, M. Coll, Mater. Res. Bull. 28 (1993) 541.
[2] J.M. García-Ruiz, A. Moreno, Acta Crystallogr. D 50 (1994) 484.
[3] J. Limpo, J. Rubio, J.L. Oteo, Boletín de la Sociedad Española de Cerámica y Vidrio 34 (1995) 77.
[4] K. Ishikiriyama, M. Todoki, K. Motomura, J. Colloid Interface Sci. 171 (1995) 92.
[5] K. Ishikiriyama, M. Todoki, J. Colloid Interface Sci. 171 (1995) 103.
[6] M. Pauthe, J.F. Quinson, H. Hdach, T. Woignier, J. Phalippou, G.W. Scherer, J. Noncrys. Solids 130 (1991) 1.
[7] V.P. Zhdanov, V.B. Fenelonov, D.K. Efremov, J. Colloid Interface Sci. 120 (1987) 218.
[8] K. Nakanishi, Y. Sagawa, N. Soga, J. Noncryst. Solids 134 (1991) 39.
[9] M.C. Roberts, K. Provost, F. Lefaucheux in: A. Ducruix, R. Giegé (Eds.), Crystallization of Nucleic Acids and Proteins. Oxford University Press, London, 1992, p. 127 (Chapter 6).
[10] H.K. Henisch, Crystals in gels and Liesegang rings, Cambridge University Press, Cambridge, 1988, p. 29 (Chapter 2).
[11] J.M. García-Ruiz, Key Eng. Mater. 58 (1991) 87.
[12] M.C. Roberts, F. Lefaucheux, J. Crystal Growth 90 (1988) 358.
[13] R.K. Iler, The Chemistry of Silica, Wiley/Interscience, New York, 1979.