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Technical Report

Automated Lab Scale Production of PVA/PEG-Enzyme Immobilisates

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Keywords: immobilisation, polyvinyl alcohol, automated production, biocatalysis, entrapment

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Abbreviations: BAL, benzaldehyde lyase, **SEM**, scanning electron microscopy, **PVA**, polyvinyl alcohol, **PEG**, polyethylene glycol

Abstract. Entrapment of biocatalysts by cryogelation is a gentle method to extend the scope of biocatalysis. To foster the use of this versatile method we devised an automated injector for the production of polyvinyl alcohol (PVA)/polyethylene glycol (PEG) beads. The device consists of a thermostated reservoir connected to a programmable injector nozzle and an agitated receiving bath for the droplets. This lab scale production unit yields up to 1500 beads with immobilised enzyme per minute with a narrow size distribution and good roundness.

1 Introduction

In the field of biocatalysis the advantages of immobilised biocatalysts were early recognised and exploited [1, 2]. Recently, it could be shown that entrapment of biocatalysts in hydrogel beads allows stable operation in otherwise deteriorating solvents [3]. Therefore, matrix entrapment of biocatalysts in natural and synthetic polymers provides a means to overcome the limitations implied by the aqueous environment which a broad range of biocatalysts require for activity and stability [4].

As entrapment matrix PVA/PEG cryogels show a number of distinct advantages [5]. They exhibit high micro- and macroporosities resulting in high mass transfer rates and show a high stability towards a wide range of compounds. The shear resistance allows application in a broad range of reactor types, which makes PVA-cryogels superior over commonly used thermoreversible gels. Furthermore the starting material is non-toxic and readily available at reasonable costs.

Entrapment in PVA cryogels is a widely applied method for the immobilisation of whole cells, and has also been described for palladium catalysts [5, 6, 7]. As isolated enzymes benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I. (EC 4.1.2.38), carbonyl reductase from *Candida parapsilosis* (EC 1.1.1.1), alcohol dehydrogenase from

Lactobacillus kefir (EC 1.1.1.2), and hog pancreas lipase (EC 3.1.1.3) have recently been entrapped and successfully applied to synthetic reactions in pure organic solvents [8, 9, 10]. Due to the mild entrapment conditions the activity yield was high even for sensitive enzymes. Generally, the stability towards organic solvents was increased by at least an order of magnitude.

PVA/PEG cryogels are most commonly applied as beads, because of the favourable hydrodynamics and abrasion stability in reactors. They are produced by cryogelation [11, 12, 13], i.e. a solution of PVA and PEG is added dropwise to silicone oil at 250 K, and gelation is induced by successive heating to room temperature [14]. Mostly, these are prepared manually. However, the highly viscous PVA/PEG solution gives rise to handling issues and large-scale production is prone to induce tenosynovitis when performed manually. The JetCutter as developed by Vorlop and coworkers was successfully employed for the production of enzyme immobilisates [15]. It is well suited for solutions with lower viscosity such as Ca-pectinate, Ca-alginate, Chitosan, gelatin and smaller bead diameters typically below 1 mm [15]. However, losses of 5-20% induced by the shear from the cutting are inevitable [16]. Lozinsky and coworkers hint towards an automated device as a Russian patent (as cited in ref. 12) apparently using pressure driven sheath flow to produce droplets but no quantitative data is accessible in literature. Again, in our experience the pressure drop over the nozzle in such setups is hard to predict or control, rising reproducibility issues.

In this study, a device for the automated production of spherical PVA/PEG cryogels for enzyme entrapment is presented. Evaluation was performed with regard to immobilisation performance, reproducibility, bead shape and size, as well as morphology of the resulting material.

2 Materials and Methods

Technical set up

Micro-Liquid-Dispenser SMLD 300G was purchased from Fritz Gyger AG (Thun-Gwatt, Switzerland). Electronical equipment was build by the university workshops of RWTH Aachen University and TU Berlin. Wacker AP 100 Silicone oil (Wacker Chemie AG, Burghausen, Germany) was used as hardening solvent for PVA/PEG solution. An Ecoline Staredition RE300 thermostat with external temperature sensor from Lauda (Lauda-Königshofen, Germany) with silicone oil M10 (Carl Roth, Karlsruhe, Germany) as coolant, was used. The sample container was thermostated by a water bath (MgW Lauda Thermo-star C3).

Preparation of PVA/PEG solution

Polyvinyl alcohol Mowiol 10-98 (polymerisation degree: 1400) and polyethylene glycol 1000 were purchased from Fluka (Neu-Ulm, Germany). A mixture of 10% (w/v) PVA and 10% (w/v) PEG was dissolved in deionised water at 90°C, 30 mg NaOH/g PVA was added at room temperature and the

solution was stirred for 30 minutes to cleave ester residues in the commercial PVA solution. The pH was adjusted to 8.0 with concentrated HCl [8].

PVA bead analysis

For analysis of size distribution PVA beads were removed from the silicone oil bath, washed with distilled water and sieved (ISO 3310/1, Retsch, Haan, Germany). Size exclusions in sieve analysis were 5.00, 4.00, 3.55, 3.15, 2.50, 2.00, 1.40, 1.00, 0.80, 0.60, 0.50, 0.40, 0.30 and 0.10 mm.

For shape analysis PVA beads were prepared by dyeing with ethanol/aqueous solution (20%, (v/v)) of 0.1% (w/v) Bromocresol green for 30 seconds. A digital picture (Olympus SP-500UZ) was converted to greyscale with maximum contrast via GIMP (Version 2). The pictures were subsequently analysed for bead roundness with the software Image Tool® (Version 3.0). The bead shape was described by roundness and compactness. Roundness is defined as $(4*\pi*\text{area})/\text{perimeter}^2$ where perimeter is the length of the outside boundary of the object. Compactness is given by $(4*\text{area}/\pi)^{1/2}$/major axis length. Both parameters are the closer to unity the rounder or closer to ideal circular the object is.

For scanning electron microscopy (SEM) PVA beads were prepared by shock freezing in liquid nitrogen and freeze drying (Alpha 1-2 LD plus, Christ, Riedstadt, Germany). SEM was conducted using a Hitachi S-2700 instrument at an acceleration voltage of 20 kV. Samples were fixed on double-sided adhesive foils and sputtered with thin gold films.

Activity assay

Benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* Biovar I [E.C.4.1.2.38] was expressed and purified using the recombinant *E. coli* $SG13009_{prep4}$ [pBAL-his_6] as described previously [17]. In the PVA/PEG solution lyophilised BAL (0.5 U mL^{-1}), thiamine diphosphate (0.1 mmol L^{-1}), and $MgSO_4$ (2.5 mmol L^{-1}) were dissolved [8]. The activity of BAL immobilised in PVA/PEG beads was determined by monitoring the BAL-catalysed carboligation of benzaldehyde to benzoin by gas chromatography (Shimadzu 2010 using a BTX column from SGE). Reactions were performed at 303 K in closed glass vessels containing 17 g of immobilisates and 10 mL of 200 mM benzaldehyde (Sigma-Aldrich, Steinheim, Germany) in *tert*-butyl methyl ether (Carl Roth, Karlsruhe, Germany).

3 Results

Set up

It was aimed for a device which would give spherical beads with narrow size distribution. Besides, thermal stress on the enzyme should be reduced to minimum. Also the influence of temperature on the cryogelation process was target of our investigations both the storage temperature and the bath temperature should be varied. The overall setup consists of the dosing unit and a thermostated bath.

The dosing unit is a double jacket tube (50 mL inner volume) with top side connection for either compressed air or nitrogen. The outlet consists of a commercially available microvalve which was embedded into an electrically heated block allowing dosage of viscous solutions at low pressures. Temperature and valve parameters were controlled electronically. Typically, the PVA/PEG enzyme solutions were transferred to the tube and pressurised to 5 kPa with nitrogen. The scheme of the dosing unit is shown in figure 1. For the investigated system the injection temperature could be kept at room temperature. Thereby, thermal stress of the enzyme was minimised.

The outlet of the valve was directed into a double jacket insulated silicone oil bath. Circulation in the receiving bath was maintained by a stirrer. The bath layout prevented the agglomeration of beads by coalescence of droplets. The distance from the valve outlet to the bath surface was adjusted to give spherical beads. Too short distances lead to the distortion of shape and a distance of 20 cm was found to give best results (Figure 1).

The PVA/PEG enzyme solution was injected into the receiving bath at 248 K. The frozen beads were collected into a dent of the bath by the induced flow. After the injection process the temperature was increased to 298 K over 500 minutes [18, 19]. Shortening to 180 minutes led to PVA beads with no apparent differences in stability, flexibility, and porosity. Higher initial temperatures of 268 K for the receiving bath were tested in accordance with literature [12, 20]. The obtained beads showed deviations from spherical

shape, presumably because freezing time is prolonged, so that beads were deformed by the stirrer.

Bead size and shape

Size of PVA/PEG beads is a function of opening and closing times of the valve. We tested three time programs at 20 ms, 40 ms, and 60 ms opening times with 20 ms, 25 ms, and 30 ms pause, respectively (Figure 2). This theoretically equates to 25, 15, and 11 beads per second allowing the formation of beads needed for a typical lab scale continuous fluidised bed reactor within minutes [21].

With 60 ms 47% of all obtained beads were within 2.5 to 3.15 mm in diameter correlating to 73% of the total mass. 83% of the mass of the beads were contained within 2.5 and 3.55 mm. At 40 ms smaller PVA beads with 80% of all the produced beads within a diameter of 2.0 to 2.5 mm were obtained. 72% of the beads showed a diameter of 2.5 mm. The shortest tested impulse length of 20 ms led to a size distribution of PVA immobilisates where 83% had a diameter between 1.4 and 2 mm. 88% of all these beads were ranged in size between 1 and 2 mm. On average the yield of beads per mass was around 75% and is due to losses in the storage vessel and at the nozzle.

So far, the production of beads is only limited by clogging after time which occurs as a result of gelation taking place at the nozzle. As the gelation process is envisaged to be induced by local high concentrations of PVA

during the thawing process favouring the formation of hydrogen bonds [13] the reason for this gel formation at the nozzle is unclear. Here, deeper understanding of the gelation process as well as the probable role of wetted materials in the nozzle is needed for further improvement.

Besides the size distribution roundness is an issue for mass transfer and robustness [22]. Bead shape analysis was performed for four bead batches. As indicator for a perfectly round shape roundness = (4 π area perimeter^{-2}) and compactness = (4 area π^{-1})^{½} major axis length can be applied. Both indicators approach unity for ideal spheres. An average roundness of 0.85±0.03 was determined. Compactness was determined as 0.95±0.02. Both parameters indicate a close spherical shape of the beads.

Gel morphology

The structure and the pore size of the PVA/PEG cryogels resulting from the automated production were determined via scanning electron microscopy (SEM) (Figure 3). Typical sponge structure and pore sizes around 1 µm are in line with previously obtained results [23]. In contrast to manually produced PVA beads gas inclusions of varying sizes were observed in 80% of the beads.

However, the activity tests with entrapped BAL showed within the error margin the same activity and storage stability as the manually produced beads. Typically a productivity of 40 µmole h^{-1} per immobilised U of BAL were obtained.

4 Conclusion

We set up a means for standardised automated PVA/PEG immobilisate lab scale production. Our method achieves high productivity of up to 1500 beads per minute. The size distribution of the PVA beads is narrow and the diameter can be varied by the valve pulse interval. The obtained beads are spherically shaped and robust allowing the use in a range of reactor configurations. Temperature stress during production of PVA/PEG immobilisates is low as temperatures are at or below room temperature. Furthermore the presented set up for automated bead production is straightforward and the additional costs are low. The used liquid dispenser is readily available. Our design for routine lab-scale production of beads now enables application of these promising immobilisates in larger scale.

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5 References

[1] End, N., Schöning, K.-U., Immobilized Biocatalysts in Industrial Research and Production, in: Topics in Current Chemistry, *Immobilized Catalysts*, Springer Verlag, Berlin Heidelberg 2004, *242*, pp. 273-317.

[2] Lalonde, J., Margolin, A., Immobilization of Enzymes, in: Drauz, K., Waldmann, H. (Ed.), *Enzyme Catalysis in Organic Synthesis,* Wiley-VCH Verlag, Weinheim 2002, pp. 163-184.

[3] Ansorge-Schumacher, M. B., Two-Phase Systems with Solidified Water Phases – Tools for Technical Use of Sensitive Catalysts. *Mini-Reviews in Organic Chemistry* 2007, *4*, 243-245.

[4] Carrea, G., Riva, S., Properties and synthetic applications of enzymes in organic solvents. *Angew. Chem. Int. Ed. Engl.* 2000, *39*, 2226-2254.

[5] Lozinsky, V. I., Plieva, F. M., Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments. *Enzyme Microb. Technol.* 1998, *23*, 227-242.

[6] Szczesna, M., Galas, E., *Bacillus subtilis* cells immobilised in PVA-cryogels. *Biomolecular Eng.* 2001, *17,* 55-63.

[7] Prüsse, U., Hörold, S., Vorlop, K.-D., Verkapselung mikroskopischer Katalysatoren in gelförmigen Polymernetzwerken. *Chemie Ingenieur Technik* 1997, *69*, 100-103.

[8] Hischer, T., Steinsiek, S., Ansorge-Schumacher, M. B., Use of polyvinyl alcohol cryogels for the compartmentation of biocatalysed reactions in non-aqueous media. *Biocatal. Biotransformation* 2006, *24*, 437-442.

[9] Metrangolo-Ruiz De Temiño, D., Hartmeier, W., Ansorge-Schumacher, M. B., Entrapment of the alcohol dehydrogenase from *Lactobacillus kefir* in polyvinyl alcohol for the synthesis of chiral hydrophobic alcohols in organic solvents. *Enzyme Microb. Technol.* 2005, *36*, 3-9.

[10] Plieva, F. M., Kochetkov, K. A., Singh, I., Parmar, V. S., Belokon, Y. N., Lozinsky, V.I., Immobilization of hog pancreas lipase in macroporous poly(vinyl alcohol)-cryogel carrier for the biocatalysis in water-poor media. *Biotechnol. Lett.* 2000, *22*, 551-554.

[11] Lozinsky, V. I., Damshkaln, L. G., Kurochkin, I. N., Kurochkin, I. I., Study of cryostructuring of polymer systems: 28. Physicochemical properties and morphology of poly(vinyl alcohol) cryogels formed by multiple freezing-thawing. *Colloid Journal* 2008, *70*, 189-198.

[12] Lozinsky, V. I., Solodova, E. V., Zubov, A. L., Simenel, I. A., Study of Cryostructuration of Polymer Systems. XI. The Formation of PVA Cryogels by Freezing-Thawing the Polymer Aqueous Solution Containing Additives of some Polyols. *J Appl Polym Sci* 1995, *58*, 171-177.

[13] Lozinsky, V. I., Cryotropic gelation of poly(vinyl alcohol) solutions. *Russ Chem Rev* 1998, *67*, 573-586.

[14] Prüße, U., Entwicklung, Charakterisierung und Einsatz von Edelmetallkatalysatoren zur Nitratreduktion mit Wasserstoff und Ameisensäure sowie des Strahlschneider-Verfahrens zur Herstellung Polyvinylalkohol-verkapselter Katalysatoren. *Landbauforschung Völkenrode* 2000, Sonderheft 214.

[15] Prüße, U., Dalluhn, J., Breford, J., Vorlop, K.-D., Production of Spherical Beads by JetCutting. *Chem. Eng. Technol.* 2000, *23*, 1105-1110.

[16] Prüße, U., Bruske, F., Vorlop, K.-D., Improvement of the Jet Cutting Method for the Preparation of Spherical Particles from Viscous Polymer Solutions. *Chem. Eng. Technol.*, 1998, *21*, 153-157.

[17] Janzen, E., Müller, M., Kolter-Jung, D., Kneen, M. M. *et al.*, Characterization of benzaldehyde lyase from *Pseudomonas fluorescens:* A versatile enzyme for asymmetric C-C bond formation. *Bioorg. Chem.* 2006, *34*, 345-361.

[18] Lozinsky, V. I., Cryogels on the basis of natural and synthetic polymers: preparation, properties and applications. *Russ. Chem Rev* 2002, *71*, 489-511.

[19] Mikhalev, O. I., Sierpinski, M., Lozinsky, V. I., Kapanin, P. V. *et al.*, Method for determination of liquid microphase volume: application to the investigation of frozen H_2O–poly(vinyl alcohol) system. *Cryo-Lett* 1991, *12*, 197-206.

[20] Leidig, E., Prüße, U., Vorlop, K. D., Winter, J., Biotransformation of poly R-478 by continuous cultures of PVAL-encapsulated *Trametes versicolor* under non-sterile conditions. *Bioproc Eng* 1999, *21*, 5-12.

[21] Ansorge-Schumacher, M. B., Greiner, L., Schroeper, F., Mirtschin, S. *et al.*, Operational concept for the improved synthesis of (*R*)-3,3´-furoin and related hydrophobic compounds with benzaldehyde lyase. *Biotechnol J* 2006, *1*, 564-568.

[22] Buthe, A., Hartmeier, W., Ansorge-Schumacher, M. B., Novel solvent-based method for preparation of alginate beads with improved roundness and predictable size. *J. Microencapsulation* 2004, *21*, 865-876.

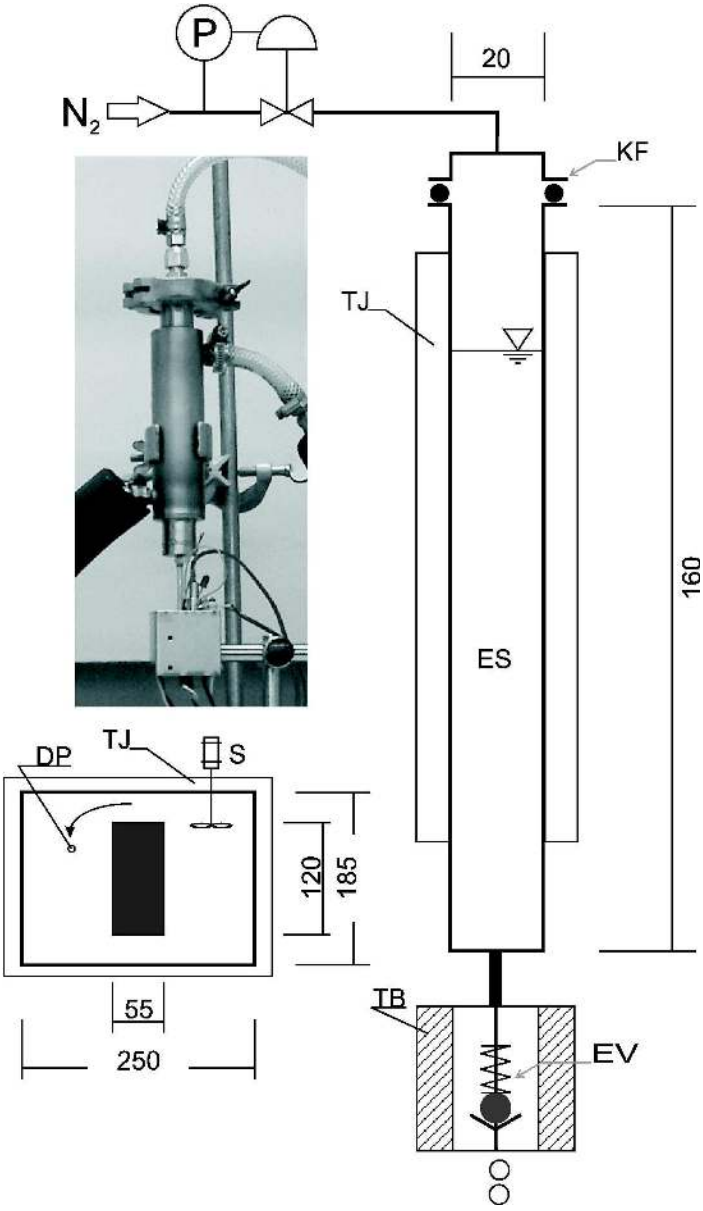
[23] Lozinsky, V. I., Vainerman, E. S., Domotenko, L. V., Mamtsis, A. M. *et al.*, Study of cryostructurization of polymer systems VII. Structure formation under freezing of poly(vinyl alcohol) aqueous solutions. *Colloid Polym Sci* 1986, *264*, 19-24.

Figure legends

Figure 1. Setup of the apparatus, right: dosing unit with electronic valve (EV) and thermostated storage for the enzyme solution (ES); middle left: photograph of the injection module; lower left: top view of the receiving bath with flow direction and drop point (DP) (dimensions in mm, KF: flange with O-ring, P: manometer, S: stirrer, TJ; thermostated jacket, TB: thermoblock).

Figure 2. Size distribution of automatically produced PVA/PEG immobilisates as a function of valve opening time (open spheres) 60 ms, (closed diamonds) 40 ms and (open triangles) 20 ms.

Figure 3. SEM images of beads (A) surface, (B-D) cross sections with increasing magnification, (E+F) surface and cross section of a bead with gas entrapment.



Setup of the apparatus, right: dosing unit with electronic valve (EV) and thermostated storage for the enzyme solution (ES); middle left: photograph of the injection module; lower left: top view of the receiving bath with flow direction and drop point (DP) (dimensions in mm, KF: flange with O-ring, P: manometer, S: stirrer, TJ; thermostated jacket, TB: thermoblock).
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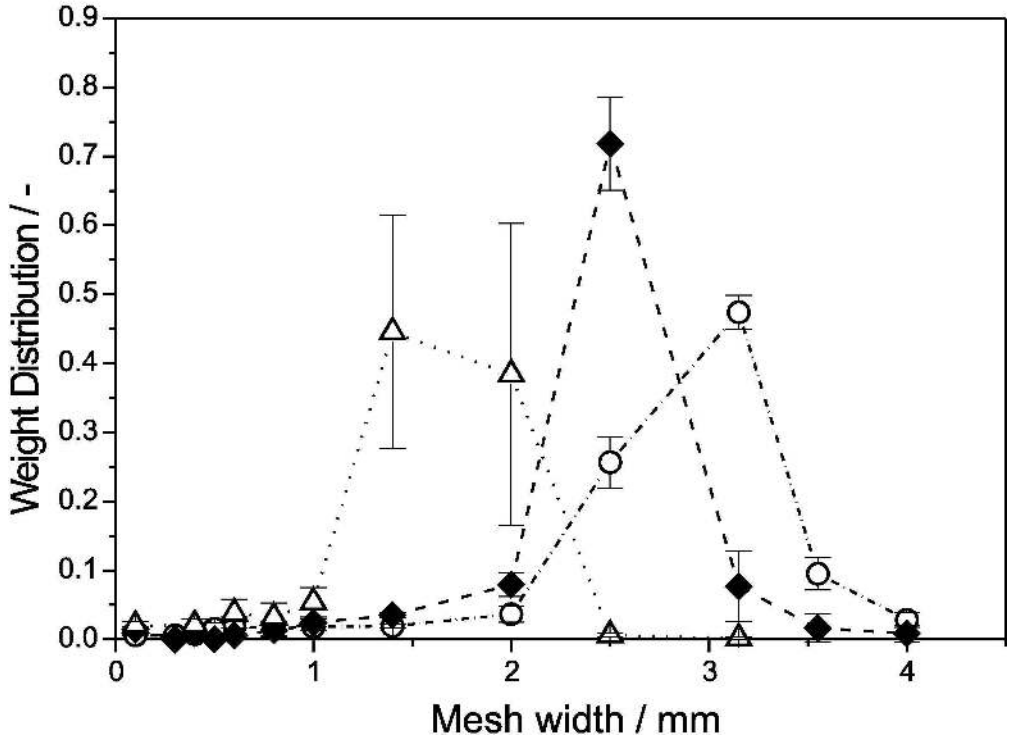

Size distribution of automatically produced PVA/PEG immobilisates as a function of valve opening time (open spheres) 60 ms, (closed diamonds) 40 ms and (open triangles) 20 ms.
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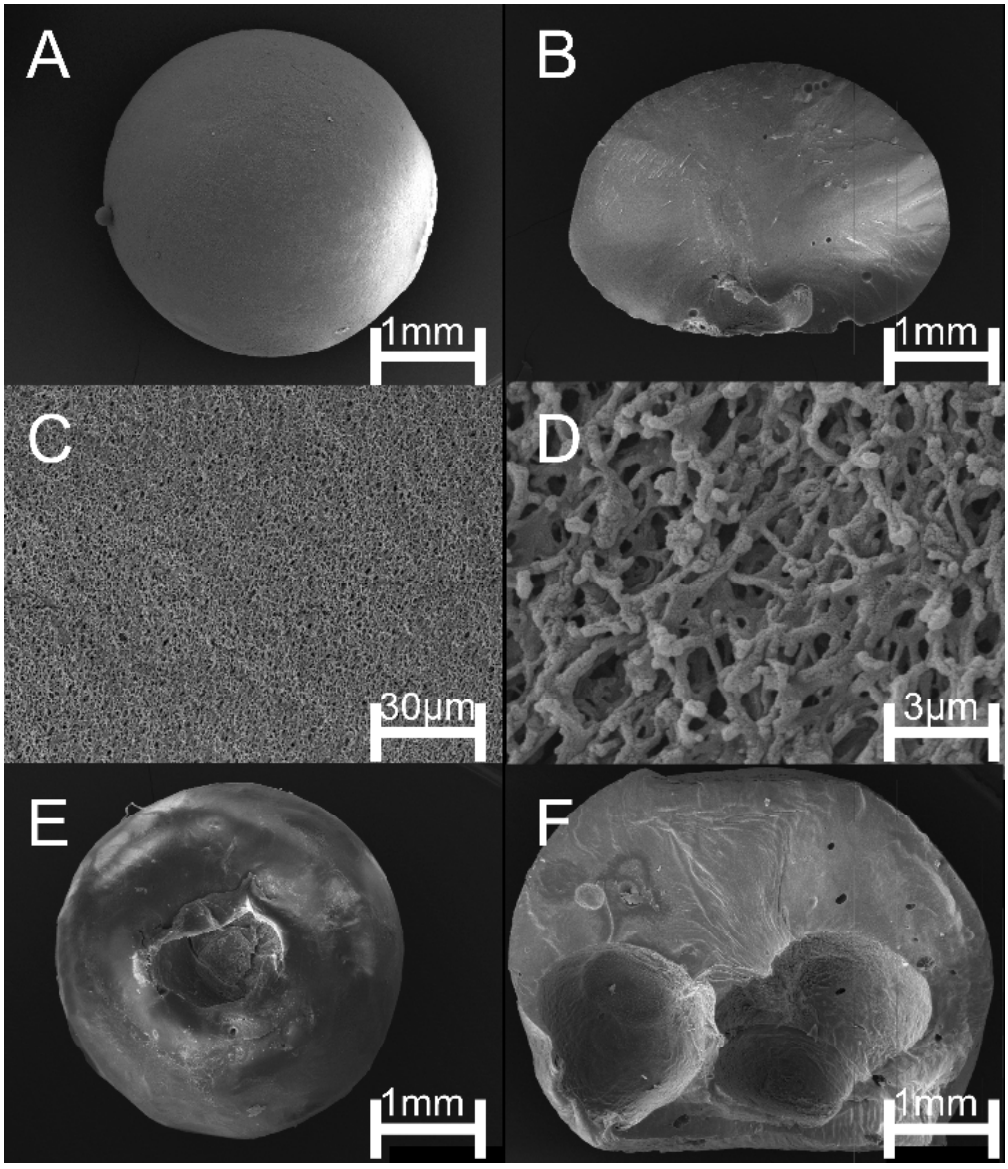

SEM images of beads (A) surface, (B-D) cross sections with increasing magnification, (E+F) surface and cross section of a bead with gas entrapment.
123x142mm (600 x 600 DPI)