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Biotech Method

Drying techniques for the visualization of agarose-based chromatography media by scanning electron microscopy

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The drying of chromatography resins prior to scanning electron microscopy is critical to image resolution and hence understanding of the bead structure at sub-micron level. Achieving suitable drying conditions is especially important with agarose-based chromatography resins, as over-drying may cause artefact formation, bead damage and alterations to ultrastructural properties; and under-drying does not provide sufficient resolution for visualization under SEM. This paper compares and contrasts the effects of two drying techniques, critical point drying and freeze drying, on the morphology of two agarose based resins (MabSelect^{TM}/d_w ≈85 μm and Capto^{TM} Adhere/d_w ≈75 μm) and provides a complete method for both. The results show that critical point drying provides better drying and subsequently clearer ultrastructural visualization of both resins under SEM. Under this protocol both the polymer fibers (thickness ≈20 nm) and the pore sizes (diameter ≈100 nm) are clearly visible. Freeze drying is shown to cause bead damage to both resins, but to different extents. MabSelect resin encounters extensive bead fragmentation, whilst Capto Adhere resin undergoes partial bead disintegration, corresponding with the greater extent of agarose crosslinking and strength of this resin. While freeze drying appears to be the less favorable option for ultrastructural visualization of chromatography resin, it should be noted that the extent of fracturing caused by the freeze drying process may provide some insight into the mechanical properties of agarose-based chromatography media.

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1 Introduction

The performance of chromatography columns in large-scale manufacturing of biotherapeutics is intrinsically linked to cost of goods and quality control. Poor understanding of the events leading to column failure can make it difficult to ensure robust operation avoiding process failures. Close examination of the bead structure can aid in this understanding, particularly of the mechanical properties of the chromatography resin. While there are various approaches and imaging techniques that can be employed for the visualization of chromatography resin, few techniques provide the level of resolution as well as the adequate representation of its three-dimensional structure required to analyze materials on the nanometer scale necessary for this.

Scanning electron microscopy (SEM) is a technique routinely used to generate high-resolution images. It is used widely in chemistry and biosciences to identify microstructures, spatial compositions and the general characterization of solid materials down to less than 50 nm in size [1]. An analogous technique also used routinely is transmission electron microscopy (TEM). It is

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Abbreviations: **CO_2**, carbon dioxide; **CPD**, critical point drying; **HIC**, hydrophobic interaction chromatography; **SEM**, scanning electron microscopy; **TEM**, transmission electron microscopy; **UCL**, University College London

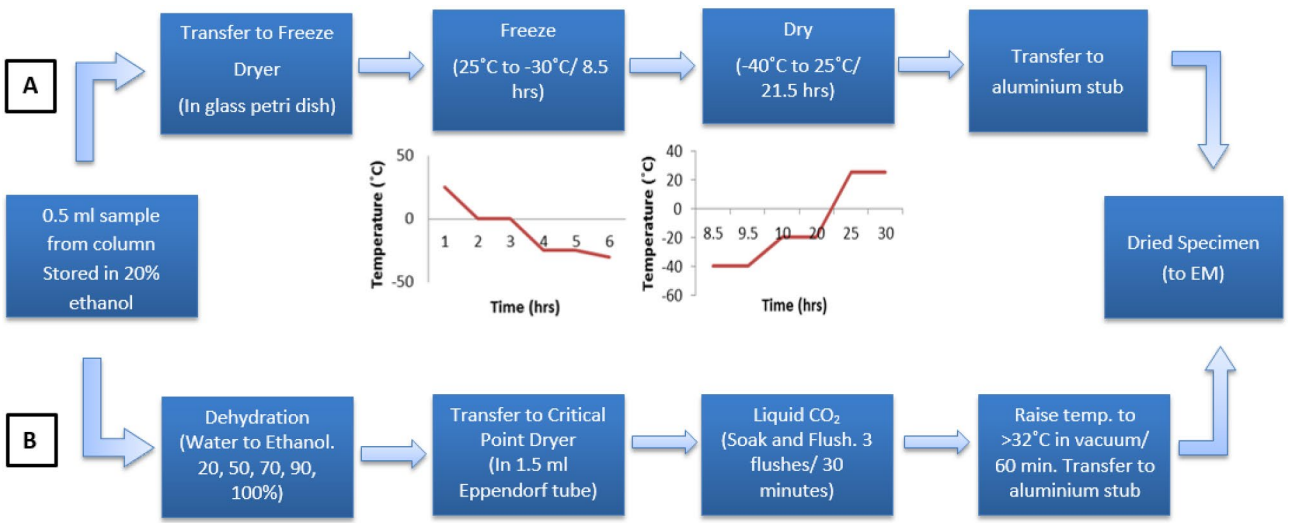

Figure 1. Selected drying conditions. (**A**) Selected freeze drying conditions. A 0.5 mL aliquot of sample is frozen over a period of 8.5 h in a graded fashion from 25°C to –30°C. The sample is transferred onto a sticky aluminium stub for SEM. (**B**) Selected critical point drying conditions. 0.5 mL of sample is dehydrated in graded fashion from 0–100% ethanol before transfer to critical point dryer where sample is flushed three times in liquid CO_2. The temperature is raised in a vacuum to above 32°C for 60 min. The sample is transferred onto a sticky aluminium stub for SEM.

able to provide higher resolution and magnification images compared to that of SEM. However, TEM only produces flat 2D images and furthermore, sample preparation is more time consuming. It involves having to finely section the sample into thin layers, embedding the sample with a fixative, staining and mounting it onto copper grids. SEM requires much less sample preparation and is able to provide more accurate information concerning the 3D morphology and the location of features relative to each other, whilst still providing sufficient resolution and magnification required for this particular application [2]. Whilst there is arguably no other instrument with the breadth of applications in the study of solid micro-materials that compares with conventional SEM, the main limitation is that samples must be dry prior to imaging. This is particularly problematic in the area of biochemistry, as most specimen require an aqueous environment to remain viable and structurally intact [3].

Drying techniques involve the removal of water from the specimen and given that water is vital in retaining the integrity of the biological specimen, an uncontrolled dehydrating process can cause substantial changes in structure and functionality. These changes can include shrinkage, cracking, breakage, morphological differences as well as other artefacts. It is therefore important to determine the drying process and the best set of controlled parameters (temperature and pressure) during this process that gives rise to the fewest artefacts in order that the images can be correctly interpreted to understand the structure.

Little has been reported on the drying technique best suited for the ultrastructural visualization of chromatography resin under scanning electron microscopy. There have been reports of the use of air drying [4], spray drying [5] as well as the water-to-absolute ethanol dehydration technique [6]. However, each one of the aforementioned techniques does not provide a sufficient level of drying for the visualization and characterization of an agarose bead on the nanometer scale. Furthermore, there have been reports that such methods do not perform well at lab-scale, in the case of spray drying [7] or lead to collapse of the beads altogether under air drying [8].

There are two established drying techniques in the biosciences that have proven to preserve biological properties of proteins and retain structure, namely freeze drying and critical point drying. Malmqvist and Hofsten (1975) [8] report the use of freeze drying for the visualization of agarose beads during bacterial degradation. Subsequently they were able to view the surface topography at low and high magnifications and from this conclude the occurrence of agarose degradation when bacteria are in direct contact with the gel. Jin, 2010 [9] reports the use of freeze drying on Sepharose (HIC) resin, whereby he was clearly able to visualize the difference in surface topography between fresh resin and lipid-fouled resin due to the increase in occultation of the pores. Both sets of research show that freeze drying may be a suitable drying technique for agarose beads.

Critical point drying has not been explored as extensively, relative to freeze drying, in terms of its application to agarose beads. However Medin et al. (1995) [10] used critical point drying to dry agarose gels, after which they went on to generate quantitative results based on pore size distributions from scanning electron micrographs. This was also later attempted by Ioannidis (2009) [11], who was able to quantify the changes in pore size and

structure of agarose beads under different ionic strengths, using scanning electron micrographs. A drawback of this method of quantitative analysis is that it employs a two-dimensional means of solving a three-dimensional problem. This may suggest some slight inaccuracies in results, which could be compared and corroborated with lab-based approaches such as that carried out by DePhillips and Lenhoff (2000) [12] who measured pore size distributions of ion exchange adsorbents using inverse size-exclusion chromatography; but nonetheless, it provides a straightforward approach towards understanding and characterizing the ultrastructure of agarose-based chromatography beads.

In this work, we compare and contrast the two drying methods, freeze drying and critical point drying, in an attempt to decipher which method allows for better understanding and characterization of agarose beads. A selected protocol is applied to both freeze drying and critical point drying (Fig. 1), as described in the methods section.

We apply scanning electron microscopy (SEM) to samples of MabSelect and Capto adhere resins as model materials to visualize the impact of critical point drying and freeze drying on both agarose-based resin types, followed by qualitative analysis of the images to assess the impact.

2 Materials and methods

Separate 1 mL slurries (75% slurry concentration in 20% ethanol) of MabSelect™ and Capto™ Adhere chromatography resins (GE Healthcare, Uppsala, Sweden) are used for this study.

2.1 Freeze drying

The protocol employed is as an adaptation of previously reported freeze drying methodologies [7, 8, 12]. In this case, the time spent in the freezing and drying stages of the process is increased. A 0.5 mL aliquot of each sample was syringed into separate sterile polystyrene petri dishes (Fisher Scientific™, 60 mm × 15 mm) and labelled accordingly. They were then placed on different shelves in the freeze dryer (Virtis Genesis 25EL, SP Scientific, US) to avoid cross-contamination. The samples were left in the freeze dryer for 30 h. The freezing stage was held for 8.5 h with a step-wise decrease in temperature, from 25°C to –30°C (1° decrease approximately every 9 min). The drying stage was held for 21.5 hwith a step-wise increase in temperature, from –40°C to 25°C (1° increase approximately every 20 min).

2.2 Critical point drying

The storage medium of our agarose beads is made up of 80% water, however water has an unfavorable critical point of +374°C and 3212 p.s.i. which will cause heat damage to the beads [13]. For this reason, the medium used for critical point drying is Carbon Dioxide (CO_2), with a critical point of 31°C and 1072 p.s.i. (http://www.leica-microsystems.com/science-lab/brief-introduction-to-critical-point-drying/). Water and CO_2 are not miscible however, and for this reason a transitional fluid is used. This fluid is miscible with both water and CO_2 and is usually an alcohol. As our chromatography media are stored in 20% ethanol, the transitional fluid used in this case is ethanol.

A 0.5 mL aliquot of each sample was syringed into a 1.5 mL Eppendorf tube and labelled accordingly. The beads were dehydrated in a graded ethanol-water series to 100% ethanol (20, 50, 70, 90, 100%) and put in critical point dryer (Polaron Critical point dryer, Quorum Technologies Ltd, Essex). In critical point dryer, the beads were flushed three times in CO_2 in a graded series for 30 min. The temperature was raised to above 32°C for 60 min.

2.3 Scanning electron microscopy

2.3.1 Sample coating preparation

A wooden spatula is used to remove the dried resin from the tubes onto the labelled 1 × 1 cm cylindrical aluminium specimen stub (Agar Scientific, UK) for sample coating. The surface of the specimen stub is sticky so that the sample is not removed during subsequent steps.

2.3.2 Sample coating

The sample is placed into the ion gun gas flow control where excess liquid is vacuumed out of the resin at 10^{-3} Pa. The Gatan, model 681 high resolution ion beam coater (Gatan, Inc., US) is then used to coat the samples in a gold/palladium alloy (1–2 nm thickness of coating). SEM imaging requires the sample to be electrically conductive in order to prevent the build-up of electrostatic charge. It is for that reason that metal objects require little preparation for SEM imaging. However samples of resin beads and other non-conductive samples tend to charge when scanned by the electron beam, consequently causing scanning faults [14]. For this reason non-conductive samples are coated with conductive metals, most commonly gold or a gold/palladium alloy. The ion beam coater is operated at 5.8 keV, angle 70°.

2.3.3 SEM imaging

After the coating process, the samples are then mounted onto an SEM carrier and inserted into the JEOL JSM-7401F field emission scanning electron microscope (JEOL USA, Inc., US) for imaging at 10.0 μA and 2 keV accelerating voltage.

3 Results and discussion

3.1 Freeze dried images

The samples imaged post-freeze drying are represented by Figs. 2(A1) and 2(A2), fresh MabSelect™ and fresh Capto™ Adhere chromatography resins respectively. These resins are widely used for the capture and intermediate stages of monoclonal antibody purification. They are made up of cross-linked agarose and it is this make up that helps in determining their mechanical properties.

Figure 2(A1) shows complete bead fragmentation, splitting the bead into two parts, of which one part can be seen in the image. Whereas, Fig. 2(A2) shows partial fragmentation of the bead; the bead appears to be partially cracked, whilst still maintaining a semblance of its spherical structure. This was a trend observed in 70% of the images collected post-freeze drying (seven out of ten beads on average) for both MabSelect™ and Capto™ Adhere resins. This is an indication that freeze drying under these conditions can lead to severe morphological changes in bead structure, as shown in both Figs. 2(A1) and 2(A2). However, what differs is the extent of fragmentation in both cases. Figure 2(A1) is completely fragmented, whereas Fig. 2(A2) is partially fragmented. This could be due to the fact that Capto™ Adhere is a stronger resin type as it is made to have a higher percentage of cross-linking of agarose media than MabSelect™ (https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314823637792/litdoc18114994_20161013165214.PDF).

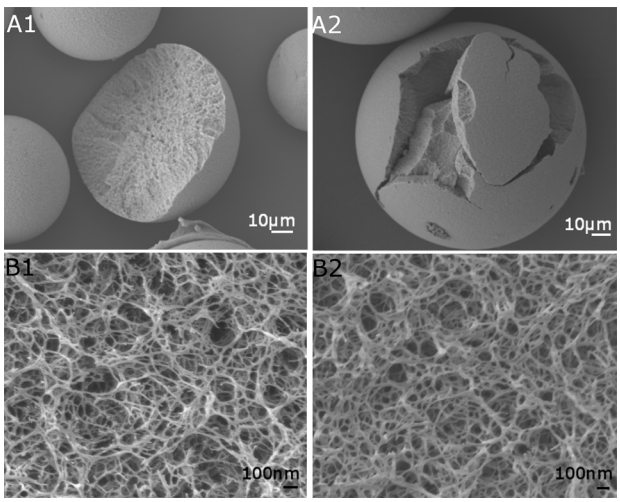

Figure 2. Scanning electron micrographs of MabSelect™ and Capto™ Adhere beads. (**A1**) Fresh MabSelect™ chromatography resin sample post- freeze drying. Whole bead image, 3.0 kV, ×950 magnification. (**A2**) Fresh Capto™ Adhere chromatography resin sample post-freeze drying. Whole bead image, 2.0 kV, ×750 magnification. (**B1**) Fresh MabSelect™ chromatography resin sample post-critical point drying. Bead surface image, 2.0 kV, ×55 000 magnification: (**B2**) Fresh Capto™ Adhere chromatography resin sample post-critical point drying. Bead surface image, 2.0 kV, ×50 000 magnification. Scale: (**A1**) 0.8 cm represents 10 µm. (**A2**) 0.7 cm represents 10 µm. (**B1**) 1 cm represents 100 nm. (**B2**) 0.9 cm represents 100 nm.

Visualization of the ultrastructure at high resolution proved difficult under these conditions. This may have been due to the changes in morphology experienced by the beads under freeze drying meaning that the surface topography was irregular, so the gold/palladium coating was not distributed evenly for clear imaging of the porous structure. An even distribution of the sputter coating is necessary to create a conductive layer on the sample. This inhibits charging, reduces thermal damage and also improves the secondary electron signal that is required for topographic observation.

3.2 Critical point dried images

The samples imaged post-critical point drying are represented in Figs. 2(B1) and 2(B2), fresh MabSelect™ and fresh Capto™ Adhere chromatography resins respectively. All micrographs showed no substantial conformational change observed with the beads under these conditions. Both Figs. 2(B1) and 2(B2) show the outer surface of the bead. These conditions allowed for high resolution images under high magnification compared to freeze dried images, meaning that the heterogeneous polymer fiber network and porous structure is clearly visible in both cases.

There are differences observed between the ultrastructure in Figs. 2(B1) and 2(B2). The matrix structure of MabSelect™ exhibits more discontinuity of fibers compared to Capto™ Adhere. This confirms the aforementioned rationale that Capto™ Adhere is a mechanically stronger agarose bead than MabSelect.

The results observed in addition to the factors for consideration associated with both drying techniques (Table 1) appear to favor critical point drying as the ideal approach for sufficient drying of agarose-based chromatography media for ultrastructural visualization under SEM. This is corroborated by observations noted by Nordestgaard and Rostgaard (1985) [14] who investigated critical point drying versus freeze drying for studies on hepatocytes. From this, they were able to conclude that critical point drying under SEM resulted in fewer morphological changes and clearer ultrastructural visualization. Whilst freeze drying requires next to no preparation or any necessary additional media, with regards to the handling of both techniques, critical point drying operates under less extreme temperature changes, which is quite critical in maintaining ultrastructure. Furthermore, critical point drying is also in the order of several hours less in time taken to carry out, which is important in the wider use of the technique and scalability. However, as indicated by the results, it must be noted that freeze drying can be applied to agarose-based chromatography media for a wide variety of applications not covered in the scope of

Table 1. Factors for consideration when choosing critical point drying or freeze drying as a drying technique for agarose-based chromatography resin

Factors	CPD	Freeze drying
Preparation	Yes (water → ethanol)	None*
Media required	Ethanol, CO_2	Water
Phases	Liquid à Gas	Solid à Gas
Time	2–3 hours**	24–30 hours**
Working temperature	32–35°C	–30/–40°C***

* Optional; Sample can be fast-frozen in e.g. liquid nitrogen prior to freeze drying
** Selected conditions
*** Can go down to as low as > –80°C for delicate sample

this research. The extreme temperatures freeze drying is capable of achieving can be used to determine the effects of other physical and mechanical factors, such as stress testing or bead deformation testing and potentially used as a bead sectioning technique for observation of the bead's interior mechanical properties using SEM. Whilst there are other imaging techniques that have been used to visualize agarose-based chromatography media, such as confocal laser scanning microscopy [15], SEM is still regarded as the most widely applied tool for high resolution imaging of bio-materials. It should be noted however, that SEM in combination with other high resolution imaging tools can provide an enhanced understanding of the overall mechanical makeup of chromatography media.

4 Concluding remarks

SEM is widely employed to visualize chromatography resins but the impact of the variety of drying methods used are given minimal examination, despite it being well recognized they have a substantial impact on the quality of the resulting images. There have been various drying techniques reported but little reported on the most suitable for clear visualization of agarose beads. Freeze drying and CPD are two techniques that are frequently used to dry biological specimen. Both techniques have been applied to MabSelect™ and Capto™ Adhere resins to determine which drying technique is best suited to the ultrastructural visualization of these beads under SEM. Under freeze drying both resins show bead breakage but to different extents. MabSelect™ shows more damage than that of Capto™ Adhere. This is expected due to the content of agarose each resin contains (MabSelect™/6% agarose; Capto™ Adhere/7% agarose). CPD provides clear ultrastructural visualization of both MabSelect™ and Capto™ Adhere without damage to the beads.

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The authors declare no financial or commercial conflict of interest.

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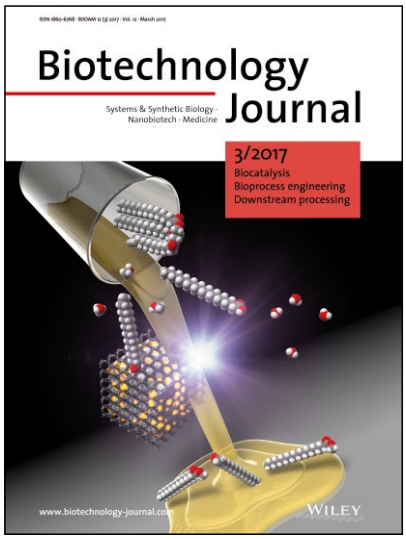

Cover illustration

The cover shows the in the one-pot conversion of free fatty acids and triglycerides, a lipase producing recombinant *Aspergillus oryzae* immobilized on matrix (sponge like matrix) can directly convert to bio-diesel from mixture of substrate. The cover is prepared by Jerome Amoah, Emmanuel Quayson, Shinji Hama, Ayumi Yoshida, Tomohisa Hasunuma, Chiaki Ogino and Akihiko Kondo authors of the article "Simultaneous conversion of free fatty acids and triglycerides to biodiesel by immobilized *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase". (http://dx.doi.org/10.1002/biot.201600400).

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Rapid Communication

Aerobic expression of *Vitreoscilla* hemoglobin improves the growth performance of CHO-K1 cells

Mariana Juárez, Claudia H. González-De la Rosa, Elisa Memún, Juan-Carlos Sigala and Alvaro R. Lara

https://doi.org/10.1002/biot.201600438

Rapid Communication

Simultaneous conversion of free fatty acids and triglycerides to biodiesel by immobilized *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase

Jerome Amoah, Emmanuel Quayson, Shinji Hama, Ayumi Yoshida, Tomohisa Hasunuma, Chiaki Ogino and Akihiko Kondo

https://doi.org/10.1002/biot.201600400