VP-SEM: Unsung Hero of SEM Imaging

Application of VP-SEM to Beat Visualization Challenges

Variable Pressure Scanning Electron Microscopy (VP-SEM) has become an important tool for the imaging of hydrated specimens and samples with low conductivity. This article outlines various research scenarios where VP-SEM allowed minimal sample processing that often enabled successive specimen reuse. We present cases where these characteristics allowed imaging of specimens otherwise impossible using conventional SEM methods.

Introduction

In the 70 years since the birth of Scanning Electron Microscopy (SEM) it has become apparent that the generic high-vacuum SEM needed to develop in respects other than increased resolution [1]. Variable Pressure (VP)-SEM makes use of gas to enhance signal detection, has charge stabilization for non-conductive samples, and further allows for thermodynamic stabilization of hydrated samples. One of the challenges of VP-SEM is that there is no simple rule that defines the parameters to use, and every specimen needs to be evaluated with its own interdependent and specimen-dependent operating parameters. In general, little or no specimen preparation is needed, and fully hydrated and out-gassing samples can be visualized since the vacuum environment (variable pressure) in the specimen chamber is separated from the ultra-high vacuum environment in the electron gun area. This is accomplished through a differential pumping system with pressure limiting apertures and turbo-isolation valves. Through my involvement in various projects it became apparent that VP-SEM often proved to be the tool to use when challenging conditions eliminated other imaging modalities for high-resolution microscopic inspection. In this article I highlight current scenarios where VP-SEM is applied as an invaluable tool to visualize sample properties.

Hydrated Biomaterials (Hydrogels)

VP-SEM with thermal control of humidity allows visualization of materials used for regenerative medical application in their in situ condition. Protein scaffolds, collagen fibres, PDMS molds, 2D matrigel and 3D polystyrene, as well as carbohydrate scaffolds of pullulan and chitosan were explored for features of
porosity and alignment, and for supporting cell proliferation (fig. 2A). Correlation with data from parallel imaging modalities give invaluable insight into dermal, cardiac and optical applications [2, 3, 4]. Drying artefacts show the importance of hydrated imaging for a proper understanding of the microenvironment in which seeded cells proliferate and grow (fig. 2B). Scaffolds are visualized unfixed and hydrated, as well as aldehyde-fixed, followed by OsO₄ contrasting, when seeded with cells. Osmication of cell-supporting scaffolds can be used with great success to enhance cell contrast for proper distinction of cell proliferation on the support surface (fig. 1).

**Large Block-Face (LBF) Sections of Resin-embedded Specimens**

In a novel Serial Section Array (SSA) SEM technique [5] we use BSE (Backscattered Electron) detection to image resin-embedded sections (70-200 nm) of biological samples that were fixed and en bloc stained with OsO₄, Tetracarbohydrazide (TCH), Uranyl Acetate and other heavy metals [6]. Serial sections on glass slides or silicon wafer are imaged over large areas without losing data through obstructing TEM grid bars or tearing of fragile formvar films. VP-SEM further enhances this technique for scanning of non-conductive surfaces, and large block-faces not suitable for the parameters of TEM grids (fig. 3). 3D reconstruction of serial images gives additional computational strength to SSA-SEM, in revealing the quantitative relationship and volumetric properties of structures.

**Non-conductive Samples with Experimental Limitation on Sputter-coating Atmosphere Sensitive Samples**

The characterization of hygroscopic and deliquescent materials (developed as scintillator detectors) using surface sensitive techniques such as electron microscopy or microprobe, demands special handling to preserve the sample surface after preparation. In a specially designed airtight container [7], equipped
with a 500 micron latex membrane and laterally positioned syringe needle, we transferred and characterized the microstructure of SrI$_2$ ceramics with VP-SEM. VP-SEM is particularly well-suited for this application as the chamber pressure can be controlled and gradually decreased, while observing the inflation, piercing, rupture and retraction of the membrane. In addition, VP-SEM did not require any further preparation or sputter-coating of the samples, which would expose the sensitive materials to air and destroy natural surface features. After retraction of the membrane the stage was raised to a working distance of 5-8 mm, pressure lowered to 10 Pa, and a 15-25 kV beam (Hitachi S-3400N) applied for resolution of exposed sample surfaces. Non-degraded surface properties are evident in samples transferred in the airtight chamber (fig. 4).

**Synchrotron Applications**

In synchrotron and other X-ray related experiments where a conductive metal coating of samples may interfere with signal detection, VP-SEM provides an excellent way to observe samples before and/or after microbeam scans. This may be needed to determine sample quality and orientation, or obtain quantitative data. In our micro-X-ray Fluorescence (XRF) experiments with *Helicobacter pylori* (fig. 5) micro-elemental composition is determined based on atomic number and bacteria are additionally immunolabelled with nanogold to locate them on the cell surface. Conventional sputter-coating is therefore prohibited since the conductive coating not only will interfere with the XRF signal from bacterial micronutrients, but also with the gold signal, and consequent localization of bacteria. Parameters of 20 Pa and 10-15 kV were used with BSE detection (Hitachi S-3400N).

**Repeated Imaging of Selected Areas Pre and Post Chemical Treatment**

VP-SEM has great potential in geological imaging, where addition of a metal layer limits the evaluation of dissolution properties and porosity at various stages of chemical treatment. Rock and gas shales (fig. 6) are evaluated repeatedly for the effect of injected fluids on microstructure over time. Using VP-SEM at 20-50 Pa and beam at 10-15 kV (Hitachi S-3400N), rock surface porosity is correlated with elastic and transport properties, and mineralogical composition [8]. Parameters are subsequently incorporated into rock-physics models of dynamic processes.

**Imaging of Valuable Devices**

Valuable samples and nanotechnological devices (fig. 7) designed for molecular and cellular application [9] can be repeatedly evaluated without an obstructive metal coating that might render further application useless. Retinal implants, physiological probes, and functional nanowire pilars and cones are some of the
structures evaluated successfully with VP-SEM. When cells are grown on such devices minimal processing can be applied (aldehyde fixation with optional OsO$_4$ treatment to enhance cell contrast) before samples are scanned wet and fully hydrated with thermodynamic stabilization. Cells can be sonicated or enzymatically removed from the surface and the device reapplied for further testing or implantation.

Conclusions

VP-SEM is often poorly understood and under-used as a tool for high-magnification imaging of specimens with experimental challenges. In all cases some degree of experimentation with sample and VP-SEM parameters is needed, and beam interaction with the specimen determined, to interpret the SEM signal output correctly. Correlating SEM imaging with other technologies, or applying different processing procedures with the same sample, can lead to a more complete picture of sample properties. It provides an excellent high-resolution imaging technology for the interface of biological and engineering sciences.

Acknowledgements

Drs M. Reichelt, R. Gaume E. Neofytou, J. Rajadas, L. Hanson, D. Bravo, S. Vialle and M. Amieva are thanked for materials and discussion, and N. Joubert for computer science support.

References


Author

Dr. Lydia-Marie Joubert
Stanford University
Cell Sciences Imaging Facility
Stanford, USA
http://taltos.stanford.edu
Contact

Stanford University
Cell Sciences Imaging Facility
94305-5301 Stanford, CA
USA