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To F-SIMS/XPS Chemical Depth Profiling of Synthetic Polymer Hydrogels

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ABSTRACT

Over the last decade the beneficial properties of hydrogels as artificial cell culture supports have been extensively investigated. Certain synthetic hydrogels have been proposed to be similar in composition and structure to the native extracellular matrix of the stem cell niche, their *in vivo* cell habitat, which is a powerful component in controlling stem cell fate. The stem cell differentiation pathway taken is influenced by many factors. When culturing cells within or upon hydrogels the choice can be strongly dependent on the underlying 3D hydrogel chemistry which strongly influences hydrogel-cell interactions.

The interrelationship between hydrogel chemistry and that of biomolecules in controlling cellular response ideally requires analysis methods to characterise the chemistry without labels and normally in 3D. Time-of-flight secondary ion mass spectrometry (ToF SIMS) has the potential to be utilised for through thickness characterisation of hydrogels. The frozen-hydrated sample format is well suited to minimise changes associated with dehydration or the complication of chemical 'fixation'. There is however significant challenges associated with this sample format. Frost formation occurs on cold samples in the ambient atmosphere affecting the quality of chemical information acquired from depth profiling frozen hydrogel samples.

We develop a simple method to remove this frost by blowing with gas prior to entry into the instrument which is shown to produce remarkably good profiles on a poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogel film where a model protein, lysozyme, is incorporated to demonstrated how biomolecule distribution

within hydrogels can be determined. A comparison of lysozyme incorporation is made between the situation where the protein is present in the polymer dip coating solution and lysozyme is a component of the incubation medium. It is shown that protonated water clusters $H(H_2O)_n^+$ where $n=5-11$ that are indicative of ice are detected through the entire thickness of the pHEMA and the lysozyme distribution through the pHEMA hydrogel films can be determined using the intensity of characteristic fragment secondary ions.

Quantitative TOF-SIMS analysis is highly desirable in biomaterial analysis as the amount and type of molecule in the material analysed may be determined. This has significant interest in hydrogel chemical analysis as cellular development on or within hydrogels may be highly influenced by the concentration and type of soluble molecule. Unfortunately, the matrix effect in SIMS changes the measured signal intensity of the analyte, preventing accurate quantitation.

For this reason, we apply X-Ray Photoelectron Spectroscopy (XPS) on the equivalent samples as the ToF-SIMS in an attempt to correlate molecular ion yields to exact elemental concentrations. Similarly to ToF-SIMS the frozen-hydrated format in XPS is however still relatively unexplored. We apply the developed preparatory procedure in 3D XPS analysis of pHEMA/lysozyme hydrogel films in a hydrated state. We show that this format is suitable for alternative high vacuum analysis techniques. Furthermore, we show that lysozyme ingress and concentration can be determined through XPS.

This work describes the first example of the characterisation of a hydrogel by ToF-SIMS and XPS in a frozen hydrated format, characterising hydrogels in a format

most reflecting its native hydrated state at culture conditions.

The described procedure allows for the mapping of biomolecules in a label free manner in 3D, furthermore allowing quantitative determination of biomolecule concentrations in hydrogels.

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ABBREVIATIONS

Abbreviation	Meaning
2D	Two dimensional
3D	Three dimensional
ASC	Adipose-derived stem cell
Ar-GCIB	Argon Gas Cluster Ion Beam
CMC	Carboxymethylcellulose
DEAAm	<i>N,N</i> -Diethylacrylamide
ECM	Extra-cellular matrix
ESCA	Electron Spectroscopy for chemical analysis
FIB	Focused Ion Beam
GCIS	Gas Cluster Ion Source
GF	Growth factor
HSC	Haematopoietic stem cell
LMIG	Liquid Metal Ion Gun
PAAm	Poly(acrylamide)
PEG	Poly(ethylene glycol)
pHEMA	Poly(2-hydroxy ethyl) methacrylate
pMMA	Poly(methyl methacrylate)
pVC	Poly(vinyl chloride)
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
ToF-SIMS	Time-of-Flight Secondary Ion Mass Spectrometry
UHV	Ultra-High Vacuum
UV	Ultra-violet
XPS	X-Ray Photoelectron Spectroscopy

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“When you are writing, you're conjuring. It's a ritual, and you need to be brave and respectful and sometimes get out of the way of whatever it is that you're inviting into the room.”

- **Tom Waits**

CHAPTER 1 – LITERATURE REVIEW

Regenerative medicine may one day revolutionise the way that the damage to the human body is treated by encouraging the human body to “self-repair”. Addressing unmet challenges in neurodegeneration, heart disease and organ donor shortages are some of the attractive promises of regenerative medicine. Advancements in stem cell biology have allowed us to increase our knowledge of tissue repair mechanisms. However, as the number of cell based therapies continues to rise, design of defined biomimetic *in vitro* culture platforms for cell expansion is highly sought after, to address the required cell numbers for regenerative treatments.

Under normal function, tissue repair mechanisms within the human body differentiate and expand cell types to repopulate the damaged area “self-repairing”. Regenerative medicine is shifting towards simulating this self-repair environment through creation of artificial substrates or ‘niches’. Stem cell niches in the human body help to tightly regulate the growth and differentiation of stem cells into their daughter cells within tissues. Stem cells are regulated by the niche architecture; simulating and controlling this microenvironment *in vitro* will unlock powerful new capabilities in directing cell fate to target growth of specific cell types for clinical treatments.

As cells naturally reside in a 3D niche within the body, the design of materials that reflect topographical and biophysical cues similar to the native *in vitro* environment are highly pursued. Traditional 2D polystyrene culture plates are unlikely to accurately reflect natural cellular environments because of their

topographical dissimilarity to the extra cellular matrix. To address this issue hydrated 3D polymer supports or hydrogels may be promising materials for *in vitro* culture^{1,2}. Since Wichterle and Lim first developed poly(ethylene glycol) methacrylate hydrogels for biological use in prostheses in the 1960s³, the number of publications concerned with these materials has increased exponentially, particularly in their biomedical applications⁴⁻⁶.

Wichterle and Lim outlined hydrogels as “chemical and mechanical properties, as well as water content that can be adjusted to meet requirements”. Hydrogels ease of chemical modification to suit encapsulated or surface supported cells is an attractive property for cell culture. User defined chemical functionalities, mechanical properties and degradability may be tailored to generate a functional biointerface and cell expansion⁷⁻⁹.

Hydrogels are regularly used as cell supports however the chemical makeup is frequently undefined, and the functionality of the material is mainly inferred on cell response¹⁰. Validation of chemical structure is prerequisite in chemistry from drug discovery¹¹ to design of inorganic catalysts¹². The lack of chemical characterisation of hydrogels is however decidedly lacking which is troubling due to their frequency of use as cell supports.

The field of mass spectrometry is concerned with chemical analysis. The surface sensitive analogue of this technique is Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). In ToF-SIMS a primary ion beam is rastered over a sample ejecting fragments relating to the surface chemistry, the fragments are measured according to the time taken to reach the detector (Time-of-Flight) and

peaks are calibrated to known masses. Previous work in surface characterization has shown ToF-SIMS to be a powerful tool in material characterization as it allows the molecular composition of the material surface to be correlated to cell response¹³, and particularly in understanding the complex surface chemistry of extracellular matrices^{14,15}. Subsurface chemical characterization of such organic systems is now possible using polyatomic sputtering beams such as Ar cluster ions beams in combination with a focused liquid metal ion analysis source.

This approach has been used on dehydrated polymer surfaces^{16,17}, however the high water content hydrogels make such samples inevitably dynamic in nature (i.e. responsive to analysis environment) as there is a natural equilibrium between the water content in the surrounding environment and within the hydrogel.

When a insoluble dehydrated polymer surface, with physical or chemical crosslinking, is introduced to an aqueous environment swelling occurs if the osmotic pressure is lower within the hydrogel as opposed to the surrounding medium¹⁸. When Introducing hydrogels into cell culture conditions, swelling and possible reorganisation of gel chemistry may occur altering the chemical groups at the surface or in the bulk gel which in turn may alter the cell-hydrogel interface and desired response¹⁹.

Biomolecules are mobile within hydrogels, including those supporting cells. The passive diffusible medium of the gel allows the movement of growth factors and excretory products of cell metabolism. Hydrogels and hydrogel microparticles for example can be loaded with highly specific therapeutic peptides or proteins²⁰. The effect of growth factors on cells is often concentration dependent.

This highlights the need to characterise 3D chemistry and map biomolecules within hydrogels.

Introducing a hydrogel in a hydrated state presents a significant challenge in ultra-high vacuum (UHV) analysis techniques such as ToF-SIMS because of the high vapour pressure of liquid water²¹. The boiling point of water is dramatically reduced at low temperatures. A pressure of 6.12 Mbar translates to a boiling point of 0.01°C. ToF-SIMS analysis requires a vacuum pressure lower than 2×10^{-6} Mbar to prevent attenuation of ejected secondary ions by background gasses and limiting surface adsorption of particles generated in the measurement. Hydrogels therefore must be introduced to ToF-SIMS in a format that resists vacuum associated dehydration if chemical characterisation in a hydrated state is desired.

Preparing a hydrogel for ToF-SIMS analysis will ideally maintain the structure present when acting upon cells as a culture support. Freezing the sample of interest through plunging into cryogenic liquid may be a way of 'fixing the gel', resisting changes associated with dehydration. Cryo-fixation of cells prior to imaging has been well established in the field of electron microscopy, however a number of consideration must be accounted for such as: crystalline ice formation in the sample resulting in fracture of delicate cellular structures associated with crystal growth and the grain boundaries, generation of topography through frosting on the sample surface, and sample dehydration through ice sublimation^{22,23}.

In this work, we explore several methods to introduce and analyse hydrogels by the ultra-high vacuum (UHV) technique of ToF-SIMS and determine the developed method's suitability for alternative UHV techniques. Furthermore, we show that biomolecules within hydrogels can be quantitatively mapped in 3D within hydrogels by applying the developed preparatory methodology for cryo-XPS analysis of frozen-hydrated hydrogels.

1.1. The Stem Cell Niche

Hydrogels are of interest to bioengineers as they present similarities to the native stem cell niche, which will be discussed in detail later. When deliberating why hydrogels and their properties are useful for *in vitro* artificial niches for cell culture, it is first necessary to define the niche, and its structure.

The idea that stem cells resided in microenvironments that regulated cell development arose from studies of transplanted hematopoietic progenitor cells almost five decades ago. Scofield *et al.* found that a stem cell is seen in association with other cells which determine its behaviour, becoming essentially a fixed tissue cell²⁴. Its maturation is prevented and, as a result, its continued proliferation as a stem cell is assured. Since then, studies of the niche have allowed us to progress in our understanding of the complex regulatory mechanisms that define cell response to their respective environments²⁵.

A stem-cell niche supporting self-renewal, restricts differentiation²⁶. A conceptualised model can be viewed as localised signalling cells and extracellular matrix that control stem cell fate in the niche. Proteoglycans and fibrous proteins within the ECM instruct cell adhesion, as well as providing chemical cues that stimulate development and maintenance of cells in the niche.

Cell-cell interactions between stem cells as well as interactions between neighbouring cells can impart control over development²⁷. The physiochemical nature of the extracellular environment also provides control mechanisms with factors such as pH, ionic strength and metabolites²⁸.

Literature frequently restricts the definition of the stem-cell niche to only include the signalling cells; therefore this conceptualised model will suffice for this discussion (*Figure 1*).

Identifying a niche can, however, be complex. Cell response may be attributed to general tissue properties rather than localised to a niche microenvironment. Ideally, a candidate niche should be transiently depleted of its full complement of stem cells and then shown to take up and maintain a newly introduced stem cell. Niches are located in multitudes of locations throughout the body: the hematopoietic²⁹, intestinal³⁰ and neural stem cell niche³¹ are examples of such specialised locations.

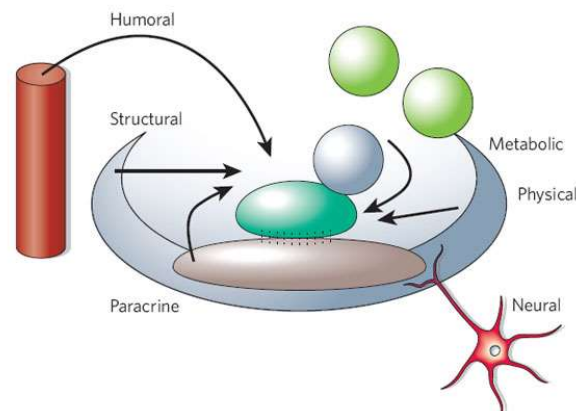


Figure 1 - The local niche environment that regulates a niche stem cell.

Metabolic cues are depicted in light green, structural and physical cues provided by the grey semi-circle, neural and humoral inputs are shown in red, the stem cell is shown in dark green at the centre of the niche.

D. T. Scadden, *Nature*, 2006, 441, 1075–9.

Stem cells in their respective niches can be thought as in a quiescent state³² 'a state of quietness or inactivity'. The balance of stem cell numbers in the niche is tightly controlled by regulatory processes, the number of cells responding to

changes in physiological demands of tissues. Upon injury or damage to the surrounding tissues, differentiation and proliferation is upregulated in response to the loss of tissue resulting in a decrease in cell number within the niche.

Feedback loops of signalling interactions provide mechanisms that instruct niche responses³³. This is visualised in (**Figure 2**) where the many potential cell fates in the niche are shown. Stem cells are inert and remain quiescent maintained by niche proteins such as cell-cell adhesion proteins (**a**). During homeostatic conditions, self-renewal divisions are asymmetric, generating one daughter cell retaining stem cell identity and another one being already partially differentiated (**b**). An example of repopulation of the niche is the reestablishment of homeostatic conditions by circulating haematopoietic stem cells (HSCs) following transplantation into host tissues irradiated to deplete endogenous stem cells³⁴. During development and/or stress the stem cell pool needs to expand via symmetric self-renewal divisions (**c**). Another possibility is the symmetric differentiation division; both daughter cells develop from a pluripotent state (**d**) further towards specific cell types.

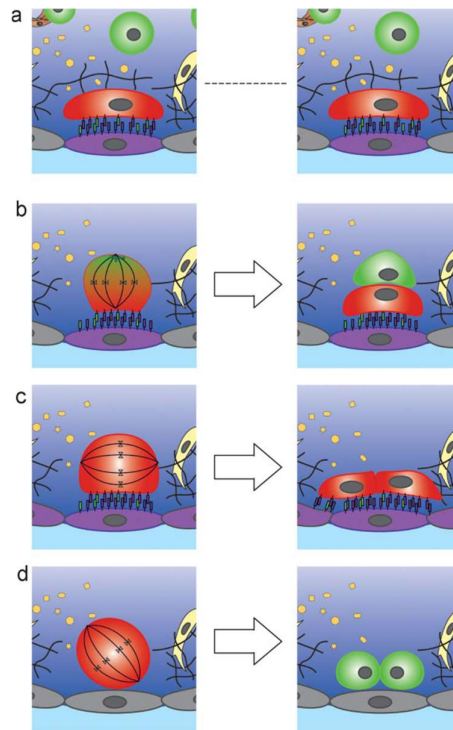


Figure 2 - The various cell fates of stem cells residing in the stem cell niche.

a) cells remain quiescent , b) self-renewal, c) expansion via self-renewal to repopulate the niche, d) differentiation into two daughter cells further to proliferate towards specific cell types.

Lutolf, M. P. & Blau, H. M. Artificial Stem Cell Niches. *Adv. Mater.* 21, 3255–68 (2009).

1.2. Artificial Niches

The extracellular matrix (ECM) is comprised of mixtures of molecules such as proteins and proteoglycans, secreted by cells which form a complex network structure with structural integrity for cell anchorage and soluble signal transmission. The ECM microenvironment is controlled by both soluble and tethered signalling cues from cell receptors. Artificial niches developed *in vitro* therefore will ideally mimic the structure and bio functions of the natural ECM. Developing artificial environments that simulates the highly specialised microenvironment experienced by stem cells *in vivo* is a fervent research area.

Stem cells respond to a variety of topographical and chemical cues provided by the underlying chemistry of the niche. Artificial niches therefore must provide a multitude of chemical and topographical cues to simulate this environment *in vitro*. Matrigel by example is a commonly used support for stem cell culture. Adhesive peptide sequences contained within structural proteins in Matrigel include laminin, entactin and heparin sulfate proteoglycans which promote cell attachment³⁵. A widely variable cell response however may be experienced with this biologically derived material³⁶ as they contain variable peptide and protein percentages³⁵. Incorporating specific physiochemical and biochemical instructors such as peptides or proteins into biorelevant synthetically generated materials in a controlled manner may provide the necessary *in vitro* niche for stem cell regulation in a xeno-free environment, addressing the variable cell phenotypic response exhibited by Matrigel usage.

1.3. HYDROGELS

Hydrogels have been present in numerous biomedical disciplines for the last fifty years, from ophthalmology in contact lenses to absorbable structures in surgery. Professors Lim and Wichtele of Prague, Czech Republic, synthesised the first hydrogel in 1955, a poly-2-hydroxyethyl methacrylate based gel. They identified the hydrogel as a revolutionary material due to its resistance to temperature, pH and tonicity conditions³⁷. Hydrophilic gels, usually referred to as hydrogels are polymer chain networks. Researchers have defined hydrogels in a variety of manners of the years³⁸, the most widely applied definition³⁹ is *“a cross-linked polymeric network produced by the simple reaction of one or more monomers”*. The other statement that is frequently ascribed to hydrogels is *“a polymeric material that exhibits the ability to swell and retain a significant fraction of water within its structure, but will not dissolve in water”*. Hydrophilic functional groups attached to the polymer backbone are the reason hydrogels have the ability to absorb water⁴⁰ (**Figure 3**).

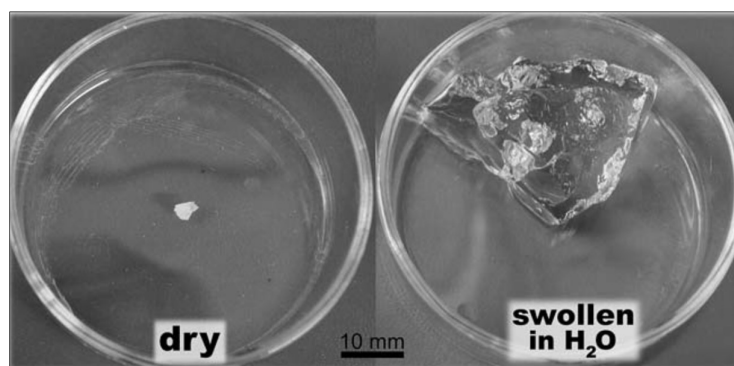


Figure 3 – Methacrylate substituted poly(phosphazene) (PMAPhos) hydrogel in dry and hydrated state.

Nykänen, S, et al. *Soft matter* 2011, 7 (9), 4414

1.3.1. CLASSIFICATION

Many materials, both naturally occurring and synthetic fit the definition of hydrogels; therefore a variety of classifications may be used to define hydrogels which are below in **Table 1**.

Table 1 – Categories used to classify hydrogels

Polymer Composition	
a)	<i>Homopolymeric</i> – Derived from a singular monomer polymerised into network
b)	<i>Copolymeric</i> – Derived from two or more different monomers, arranged in a random, block or alternating configuration
c)	<i>Multipolymeric Interpenetrating Polymeric (IPN)</i> - Made from two independent cross-linked synthetic and/or natural components
Configuration	
a)	<i>Amorphous</i> (non-crystalline)
b)	<i>Semi</i> : a complex mixture of amorphous and crystalline phases
c)	<i>Crystalline</i>
Cross-linking Method	
<i>Physical networks</i> have transient junctions that arise from either polymer chain entanglement, whilst <i>covalent networks</i> have chemical linkages between the chains	
Physical Appearance	
<i>Film, microsphere or other gelated shape</i>	
Network Electrical Charge	
a)	<i>Nonionic</i> (neutral)
b)	<i>Ionic</i> (including anionic or cationic)
c)	<i>Amphoteric</i> (ampholytic) containing both acidic and basic groups
d)	<i>Zwitterionic</i> containing both anionic and cationic groups in each structural repeating unit

1.3.2. SYNTHESIS & PREPARATION

A variety of hydrogels are used in the biomedical field: Natural polysaccharides⁴¹ (agarose, alginate, gellan), proteins⁴² (collagen and gelatin), and synthetic polymers such as poly(ethylene glycol)⁴³ (PEG) and poly (hydroxyethyl methacrylate)⁴⁴ show usage as cell supports. Additional examples are shown in **Table 2.**

Water-soluble linear polymers both natural and synthetic way be crosslinked in a number of ways

1. Crosslinking polymer chains via a chemical reaction
2. Ionising radiation to generate main-chain free radicals which can cross-linked at junctions
3. Physical interactions such as entanglements and crystallite formation.

Gelation, the process of forming gels involves the linking of macromolecular chains leading to progressively larger branched polymers⁴⁵. Increased polymer size through continuous linking has a negatively proportional effect on solubility. Generally hydrogels may be prepared from either synthetic polymers or natural polymers. Simply, a hydrogel is a hydrophobic network cross-linked in some fashion to produce an elastic structure. Any technique which can be used to create a cross-linked polymer can be used to produce hydrogel. A common method in production of hydrogels involves employing free-radical polymerisation/copolymerisation by reacting monomers with cross-linkers⁴⁶.

Table 2 – Examples of some natural and synthetic polymers used in biologically relevant hydrogels

Natural Polymers and their Derivatives
<i>Anionic polymers: Hyaluronic acid, Alginate acid, Pectin, Carrageenan, Chondroitin sulfate, Dextran sulfate</i> <i>Cationic polymers: Chitosan, Polylysine</i> <i>Amphipathic polymers: Collagen, Gelatin, Carboxymethyl chitin, Fibrin</i> <i>Neutral polymers: Dextran, Agarose, Pullulan</i>
Synthetic Polymers
<i>Polyesters: PEG-PLA-PEG, PEG-PLGA-PEGm PLA-PEG-PLA, PHB</i> <i>P(PF-co-EG)±acrylate end groups</i> <i>P(PEG/PBO terephthalate)</i> <i>PEG-bis-(PLA-acrylate), PEG±CDs, PEG-g-P(AAm-co-Vamine)</i> <i>PAAm, P(NIPAAm-co-AAc),</i> <i>P(NIPAAm-co-EMA), PVAc/PVAm PNVP, P(MMA-co-HEMA), P(AN-co-allyl sulfonate)</i> <i>P(biscarboxy-phenoxy-phospazene), P(GEMA-sulfate)</i>
Combinations of Natural and Synthetic Polymers
<i>P(PEG-co-peptides), alginate-g-(PEO-PPO-PEO), P(PLGA-co-serine), collagen-acrylate, alginate-acrylate,</i> <i>P(HPMA-g-peptide), P(HEMA/Matrigel), HA-g-NiPAAm</i>

1.3.2.1. BULK POLYMERISATION

Monomers with unsaturated regions within the structure, i.e. double bonds may be used for the production of hydrogels. The pi bond is susceptible to radical attack, initiating polymerisation. Many vinyl monomers may be used to produce a wide array of hydrogels⁴⁷. The wide variety of monomers enables one to prepare the hydrogel with desired physical properties for a given application. Usually a small amount of cross-linking agent is added in any hydrogel formulation. The polymerisation reaction is normally initiated with radiation, ultraviolet, or chemical catalysis. Bulk polymerisation is the simplest technique and only involves monomer and monomer-soluble initiators. High rate of polymerization and degree of polymerisation occurs due to the high concentration of monomer. Free radical polymerisation of vinyl based polymers is a powerful tool in biomaterial synthesis, allowing access from flexible elastomers to strong polymer fibres⁴⁸. Sorbiton methacrylate gel prepared by free radical polymerization have been described as tissue engineering scaffolds⁴⁹. Examples of other hydrogels produced by this method include: *N*-isopropylacrylamide⁵⁰, poly(acrylic-co-vinylsulfonic)⁵¹ acid 2-Acetamidoacrylic acid⁵².

1.3.2.2. SOLUTION POLYMERISATION/CROSS-LINKING

In solution copolymerization/cross-linking reactions, the ionic or neutral monomers are mixed with the multifunctional cross-linking agent. The polymerization is initiated thermally by UV-irritation or by a redox initiator system. The presence of solvent serving as a heat sink is the major advantage of the solution polymerization over the bulk polymerisation. Advantages include: rapid curing time, low energy requirements, use of room temperature and solvent free formulations as well as low cost⁵³. Novel *N,N*-diethylacrylamide (DEAAm) copolymer hydrogels have been reported as potential drug delivery agents in implantable matrices⁵⁴. Functional hydrogel composite films for endothelial and osteoblast cell co-culture have been reported⁵⁵, including benzophenone moieties in the crosslinking matrix which was shown to facilitate cell growth, and simultaneously enable partial degradation stimulating cell proliferation. Chitosan-sulfate hydrogels prepared by photocrosslinking have been reported to promote increased proliferation of vascular endothelial and smooth muscle cells compared to purely chitosan hydrogels⁵⁶.

1.3.2.3. POLYMERISATION BY IRRADIATION

Ionizing high energy radiation, like gamma rays and electron beams, has been used as an initiator to prepare the hydrogels of unsaturated compounds. The irradiation of aqueous polymer solutions results in the formation of radicals on the polymer chain^{57,58}.

1.3.2.4. SUSPENSION POLYMERISATION OR INVERSE-SUSPENSION POLYMERISATION

Dispersion polymerisation is an advantageous method since the products are obtained as powder or microsphere beads. The cooling process of hot gelatine solutions initiates Helix-formation forming junction zones. An opposite thermal effect can be observed where a hydrogel can be obtained by heating polymers; polyethylene oxide-polypropylene oxide⁵⁹ and polyethylene glycol-poly(lactic acid)⁶⁰ hydrogels can form by this process. Polyelectrolyte solutions can be gelled by the addition of a multivalent ion of the opposite charge, one example is the synthesis of chitosan-dextran hydrogels by addition of di- or tri-valent counterions⁶¹. Coacervation involves employing a mixed polymer solution with opposite charges on the basis that polyanions and polycations will stick together to form soluble and insoluble complexes. Hydrogels have been produced by coacervation of polyanionic xanthan with polycationic chitosan⁶².

Carboxylic functional groups are inherently in equilibrium between charged and uncharged states in a neutral solution. Lowering the pH of an aqueous solution of polymers with carboxylic functionalities shifts the equilibrium towards protonated carboxylic groups and initiating intermolecular hydrogen bonding. Hydrogen-bound carboxymethylcellulose (CMC) hydrogels have been described by this method⁶³ by dispersing CMC into HCl. Hydrogen bonding induces the now bound matrix to drop out of solution due to the change in solubility. Aggregation of proteins can be explored as a method of forming hydrogels. Heat induced treatment of compounds such as gum arabic⁴ results in aggregation of the proteinaceous components, increasing the molecular weight and enhances the mechanical properties with water binding capacity.

1.3.3. PROPERTIES

Softness, smartness in terms ease of functionalisation, and ability to store water has resulted in consideration of modular hydrogel networks for artificial niches. Some physiochemical aspects of the natural tissue are simulated when culturing cells on hydrogels, as they provide a fluidic structural support able to passively transport nutrients and excretory products.

Hydrogels exhibit physiochemical properties that can be easily adjusted, e.g. functional group chemistry, electrical charge, pore size, viscoelasticity and stiffness. A number of important physiochemical parameters of hydrogels are shown in **Table 3**.

Table 3 - Important physiochemical parameters and properties of hydrogels

Type of Hydrogels
Physical gel
Chemical Gel
Molecular Structures
Linear polymers
Block copolymers
Graft copolymers
Interpenetrating networks (IPNs)
Polyblends
Composition of Hydrogel
Natural polymers and their derivatives
Synthetic polymers
Combinations of natural and synthetic polymers
Important Properties
Degradable or non-degradable
Injectable or pre-fabricated
Mechanical strength
Ease of handling
Shape and surface/volume ratio (sheets, cylinders, spheres)
Closed vs. open pores
Water content and character
Chemical modification (e.g. having attached cell adhesion ligands)
Added bioactive components (cells, drugs)
Sterilizability

1.3.4. SWELLING & HYDRATION

The modulus of elasticity measures an object or substance's resistance to being deformed elastically when a force is applied to it. The elastic modulus of an object is defined as the slope of its stress–strain curve in the elastic deformation region.

A stiffer material will have a higher elastic modulus^{4,51}. The dependence of the volume of water or swelling capacity in hydrogels on elasticity has been well established^{64–66}. The elastic modulus of the prepared hydrogel largely dictates equilibrium swelling. The degree of cross linking and charge densities of the polymer network define the elastic behaviour.

Illustration of which may be found below in *Figure 4*. The elastic modulus of poly(acrylamide) (PAAm) hydrogels and cross linking efficiency after the gel preparation is plotted against percentage of cross linker (MBAAm) content for three series of gels prepared at various monomer concentrations. The figures show the elastic modulus of the gel increases with the percentage of cross linker, as more bonds are formed in the PAAm hydrogel, increasing the strength of the gel.

A higher initial monomer concentration results in a greater elastic modulus as the percentage of available groups for cross-linking reactions increases. For higher initial monomer concentrations, the cross-linking efficiency (ϵ_{xl}) decreases as the availability of cross-linkable groups in the pAAm chain is far in excess of the percentage of radicals generated by the MBAAm.

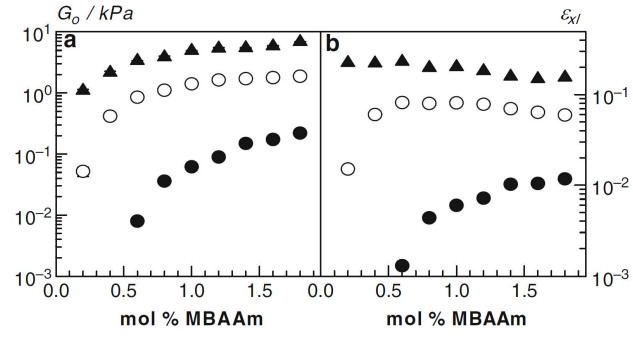


Figure 4 - Dependence of crosslinking on pAAM hydrogel elasticity

a) The elastic modulus (G_0) and (b) the cross linking efficiency (ϵ_{xl}) shown as a function of MBAAm concentration. Initial monomer concentration $C_0 = 3$ (filled circle), 5 (open circle), and 7 w/v % (filled triangle).

1.4. Hydrogels as Artificial Niches

Artificial cell culture supports will ideally be able to provide the same instructive cues present in the natural *in vivo* stem cell niche. Hydrogels derived from natural sources such as collagen or hyaluronic acid provide bioactive and biocompatible proteins that promote processes such as facilitating focal adhesion, however natural hydrogels present issues with batch-to-batch variability and difficulty in tuning features³⁶.

In vitro cell culture on artificial substrates involves creating a structural environment with biomaterial interactions and bio-signals that promote cellular adhesion and growth. Hydrogels have interest as artificial matrices due to exhibiting physiochemical properties that can be easily adjusted, e.g. electrical charge and pore size. Mechanical properties such as viscoelasticity and stiffness can also be modified to impart control. Large amounts of water or biological fluid are able to be absorbed due to interconnected pores, similar to tissues in the body.

Desired mechanical properties can be achieved by choice of polymer used, and their concentrations with crosslinking density. Combinations of natural and synthetic hydrogels can be utilised; desirable mechanical and biocompatible properties are due to the coexistence of both synthetic and biological components. For these reasons hydrogels have shown significant interest as artificial niches for *in vitro* cell culture^{67–71}.

The native ECM contains signalling molecules such as growth factors (GFs), which trigger a wide range of cellular responses in tissue development. Artificial hydrogel matrices must therefore have the ability to deliver growth

factors in a controlled manner. One such method is by direct encapsulation within the matrix⁷². Enhanced chondrogenesis has been described in adipose-derived stem cells (ASCs) in chitosan-based hydrogels by coencapsulating microspheres containing either BMP-6 or TGF- β 3⁷³ to allow delivery of growth factors in a controlled manner, similar to in their *in vivo* niche.

Most stem cell types require anchorage, thus needing adhesion to a matrix and/or other cells for maintenance of potency⁷⁴. Covalent tethering of proteins and peptides to hydrogels has been performed to provide the necessary adhesive cues that facilitate cell attachment has been reported, and example of which included the functionalisation of PEG hydrogels with Heparin, stimulating and sequestering BMP2 to promote hMSC osteogenesis⁷⁵.

1.5. Chemical Tuning of the Hydrogel-Cell Bointerface

Cells phenotypic response to substrate may be strongly influenced by the type and amount of cell responsive moiety on the surface. Peptides such as RGD and IKVAV have been widely employed to promote cell adhesion in a controlled manner^{76–78}. Similarly modifying hydrogels with specific surface or 3D chemistries may allow cell phenotype to be controlled. This is aptly illustrated by *Benoit, D.S. et al's* paper on “*Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells*”⁹ (**Figure 5**).

Photoencapsulation of hESCs in a variety of methacrylate based derivatives was shown to have dramatic effects on cell development, charged phosphate groups leading to osteogenesis and hydrophobic t-butyl groups inducing adipogenesis⁷⁹. This example speaks to the versatility of polymer materials in terms of ease of functionalisation. It is however vital the chemistry experienced by the cell is determined, to allow for assessment of the specific chemistry on the cell phenotype.

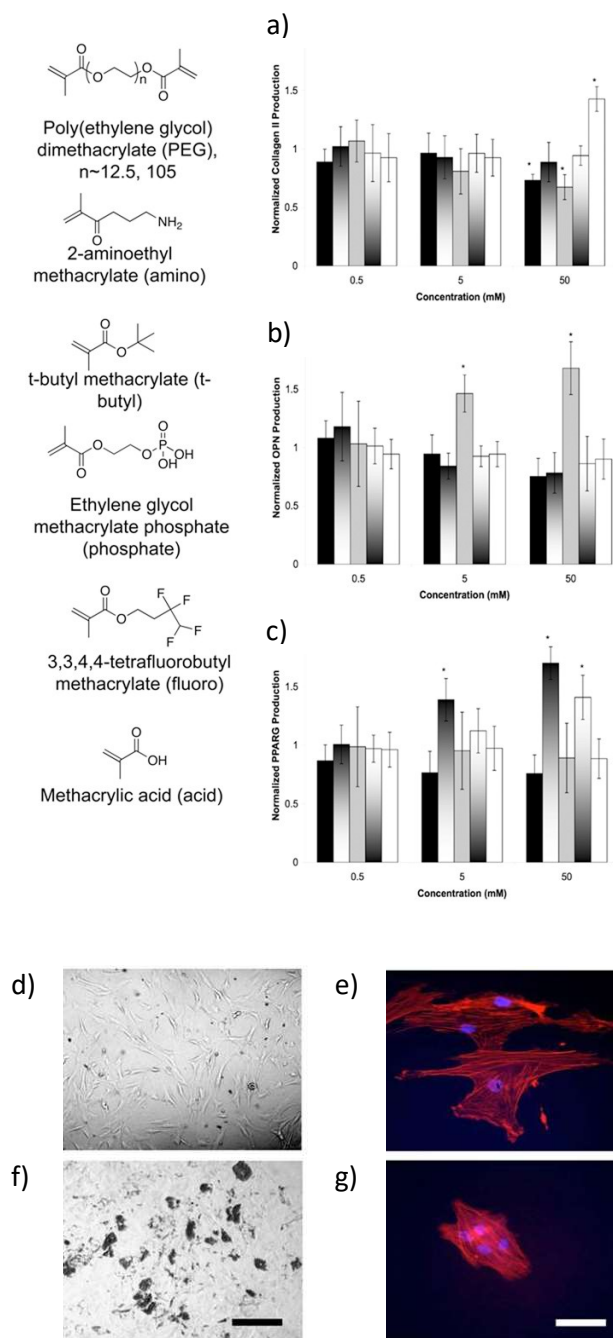


Figure 5 - hMSC morphology in response to surface chemistry.

a-c) Chemical structures of functional moieties incorporated (left column) and protein expression of hMSC (right column, as measured by immunostaining) collagen a), OPN (b), and PPARG (c) after 10 days of culture in control media on (left to right) unmodified PEG, Amino, t-Butyl, Phosphate Fluoro and Acid surfaces. d-g) Representative light (left) and fluorescent (right) micrographs of TRITC-Phalloidin (red) and DAPI (blue)-stained of hMSC depicting morphology cultured on Phosphate (d and e), and t-Butyl (f and g)-functionalized surfaces (bar = 200 μm for light micrographs, bar = 50 μm for fluorescent micrographs).

Benoit, D.S. W. et al. *nat. mater.* 7, 816–23 (2008).

1.6. Inference of Cellular Response

Cellular response to hydrogels may be highly influenced by the chemical composition of the hydrogel. It is not uncommon in literature that successful modification is inferred mainly on cell response to the material rather than measured directly⁸⁰. Advancements in structure determination in synthetic chemistry have evolved due to the need for structure validation. When considering biomaterial supports such as hydrogels, the same stringent requirements of structure elucidation are surprisingly lacking. Determination of physical properties such as surface roughness and elasticity may be gained through optical techniques such as scanning electron microscopy (SEM) or transmission electron microscopy (TEM) and profilometry respectively. However chemical analysis of hydrogel is still a complex issue.

This is largely due the significant challenges of chemical measurement of polymer supports in hydrated environments, of which we address in this work.

1.7. ToF-SIMS

Time-of-flight secondary ion mass spectrometry is a powerful technique used to gain chemical information from surfaces. Used in materials science and surface sciences, a focused ion beam is used to sputter the sample surface, upon which secondary ions are ejected. The particles may be electrons, ions, neutrals or photons⁸¹.

Time-of-flight (ToF) refers to the detection method with which the ionised particles are analysed. The ionised species are ejected into a “flight tube” post bombardment. Secondary ion pulses are accelerated to a given potential. The heavier ions travel more slowly, therefore the measured time increases with mass of the secondary ion. A spectrum of the secondary ion intensities is generated, and calibration may be performed to particular secondary ions of known mass/charges. A depiction of a typical ToF-SIMS set up is shown below in

Figure 6.

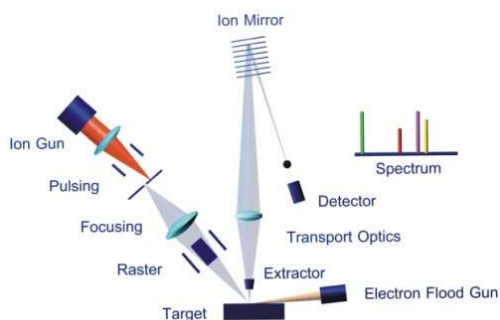


Figure 6 - Schematic of experimental ToF-SIMS set up

ION-TOF, Gesellschaft für Herstellung von Massenspektrometern mbH, Heisenbergstraße 15, D-48149, Münster, Germany

When the high energy beam of ions, particles etc. hits the surface, a billiard-ball type collision occurs between the surface atoms and the bombarding species. A 'cascade' of collisions results in emission of ionized atom clusters or fragments⁸². Studying ion fragments of surfaces can give important qualitative information on functional group distribution throughout materials. Proteins and peptides on various substrates have been characterised by ToF-SIMS, studying the ion fragments originating from their constituent amino acids^{82,83}.

Gaining chemical information on surface makeup is not without its problems. Topography is a key issue in SIMS as accentuated structures on the sample surface induce distorted elemental distributions in the measurement. The origin of this effect is the 45° incidence angle of the analysis beam, recording planar images with distortion of the sample surface. Analysing surfaces encountered in the chemical and biological field may present challenges as topography may be pronounced. Certainly, this is the case in ToF-SIMS imaging of tissue or cells⁸⁴. Uneven sample topography may have significant impact on mass resolution, i.e. ability to distinguish secondary ions with similar masses in the calibrated chemical spectra as a result of peak broadening.

In ToF-SIMS a liquid metal ion gun (LMIG) is typically employed to generate a high energy pulsed ion beam (e.g., Bi⁺ or Bi₃⁺ at 25 keV and 0.1 pA) which can be focused to around 150nm for analysis. An emphasis in recent years has been on using larger polyatomic ion sources such as gold clusters⁸² or buckminsterfullerene⁸⁵; as these ions open up the potential for higher yields of higher mass fragments. For complex samples large secondary ion yields normally contain greater chemical

information than smaller ones, high sensitivity is desirable as key components can often be present in low amounts. Larger polyatomic projectiles also have the added benefit of depositing their energy near to the surface, limiting damage to the underlying material.

Static SIMS gives qualitative information on surface chemical composition. If the primary ion beam is used in a continuous mode, erosion of the sample surface can occur; this is dynamic SIMS or 'depth profiling'. Depth profiling in this mode is problematic. As the surface is impacted by the primary ion beam, damage occurs through fragmentation in the area rastered. A second impact in this area will result in collecting secondary ions that have been influenced by ablation of the initial ion beam. This is a factor of the greater depth penetration of small, high energy projectiles, and results in a loss of secondary ion signal and molecular specificity. Lowering the energy of ion beam reduces sub surface damage, however reduces the yield of secondary ions, which may be problematic for detecting markers specific to sub-surface components of interest if they are present in low amounts.

If the energy of the projectile is limited to the surface region, then the possibility of analysing undamaged material beyond the surface region becomes possible. This may be achieved through usage of a cluster ion beam, in parallel with a LMIG. ToF-SIMS depth profiling is frequently performed in the dual beam mode, where the analysis sequence is decoupled from the etching sequence by alternating two or more ion beams⁸⁶. Etching is performed using a high current but low energy

source (e.g., C_{60}^+ at 10 keV and 1 nA)^{84,87} whilst analysis is performed by a high spatially resolved primary ion beam such as Bi^{n+} .

C_{60}^+ as an etching beam suffers from deposition of carbon on the bombarded surface, as the cluster energy and carbonaceous C_{60} fragments deposit on the material surface. Argon, as an inert gas desorbs upon impact therefore contamination of analysed surface does not occur^{88,89}. This dual beam approach combines the high mass and lateral resolutions of LMIG beams with the lower energy cluster beams that induce less damage and mixing, leading to an improved depth resolution.

1.7.1. THE PROCESS OF SPUTTERING

Rastering a primary ion beam over the sample, be it bismuth, caesium or other ion source results in the emission of atoms and atom clusters which are ionised in the course of leaving the surface⁹⁰. The majority of the energy of the incoming primary ion beam is absorbed by the material, however a small minority of sputtered secondary ions are ionised are ejected. A depiction of this is shown below in

Figure 7.

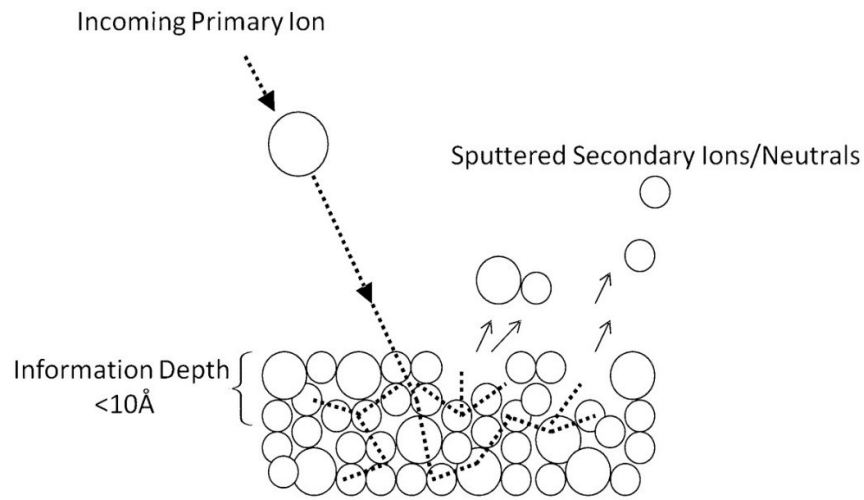


Figure 7 - Primary ion Impact on a surface

J.I. Denbigh and N. P. Lockyer, mater. sci. technol., 2015, 31, 137–147.

Particle emission from the surface is consequently affected by the electronic state of the matrix. This phenomenon, termed the matrix effect, presents complications for quantitative analysis, as yield of secondary ions are strongly influenced by the electronic state of the material being analysed⁹¹.

The secondary ion current of species m , I_s^m is related to the primary ion particle flux, I_p , the sputter yield of species m , y_m , the ionization probability of positive or negative ions, $\alpha^\pm$, the fraction concentration of θ_m in the surface layer and the transmission of the analysis system, n .

$$I_s^m = I_p y_m \alpha^\pm \theta_m n$$

Equation 1 – secondary ion current

Vickerman, j. c.; Gilmore, I. S. Surface Analysis: The Principal Techniques; 2009.

The optimum energy for sputtering beams occur around 20 to 40 KeV with the ionisation probability tending to maximise with energy at around 5 to 50 keV. Above this energy, the primary beam penetrates deeper into the material and results in less energy returning to the surface. Generally, the larger the mass of the bombarding particle, the closer to the surface the energy will be deposited and hence the greater will be the yield. If the material analysed is a polymer, impact will inevitably destroy part of the monomer unit. The concept of damage cross section is therefore useful. Damage cross-section determines the average size of the surface area damaged by one single primary ion bombardment. In the case of polymers sputtered using monoatomic beams which form cross links, there is decay in the intensities of characteristic fragment ions. It is usually observed that the larger the fragment, the greater the rate of damage⁹². The damage cross section I_m is related to the original surface density of the respective molecular species, I_{mo} , and the exponential of the secondary ion intensity σI_p .

$$I_m = I_{mo} \exp(-\sigma I_p)$$

Equation 2 – damage cross section

Vickerman, j. c.; Gilmore, I. S. Surface Analysis: The Principal Techniques; 2009.

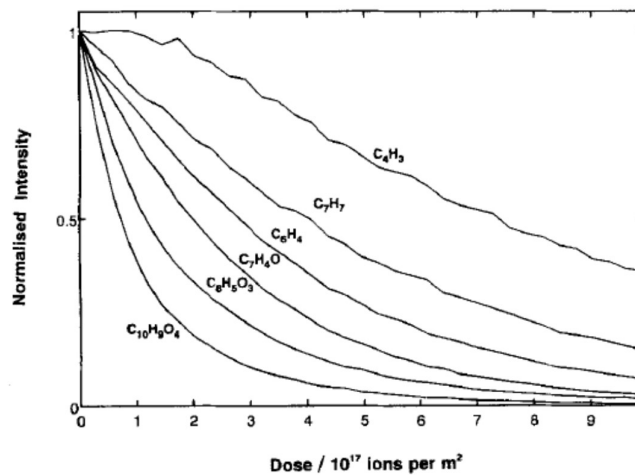


Figure 8 - Normalised damage curves for selected masses from PET sputtered using C₆₀⁺

I. S. Gilmore and M. P. Seah, 1996, 24, 746–762.

1.7.2. THE STATIC LIMIT AND DEPTH PROFILING

Following ion bombardment all molecules within 5-10 nm from the point of impact are damaged, and no more molecular information can be obtained from this zone⁹³. The amount of secondary ions available to be analysed is therefore limited. If the primary ion dose is limited so statistically each impact hits a fresh, unrastered area, the SIMS spectrum reveals molecular information of the material ablated through its peaks and peak pattern. As the ion dose increases, the molecular signal decreases then vanishes when the whole area has been damaged. The damage cross-sections for organic materials are in the area of $5 \times 10^{-14} \text{ cm}^{-2}$. This means around 50% of the signal intensity would be lost after a primary ion dose of $10^{13} \text{ ion cm}^{-2}$. This ion dose is considered as the **static limit** for analysis. Proceeding beyond this ion dose would suitably damage the underlying material. This is well documented in literature, Russo *et al* demonstrated the impact of increasing ion dose on water ice films with C_{60}^{+} and Au_3^{+} with deeper penetration of energy deposition as a function on increased ion intensity (**Figure 9**). As mentioned the static limit prevents continual analysis of the surface, as the high damage cross sections observed from analysis beams result in a loss of ion signal. For 3D analysis of materials sputter ion beams must be applied along with the analysis beam to etch and analyse the surface in question. Indeed, depth profiling is frequently reported in such mode^{23,84,94,95}, where the analysis sequence is decoupled from the etching sequence by alternating two or more ion beams. This dual beam approach combines the high mass and lateral resolutions of LMIG beams with the lower energy cluster beams that induce less damage and mixing,

leading to an improved depth resolution compared to usage of monoatomic ion beams as etching sources.

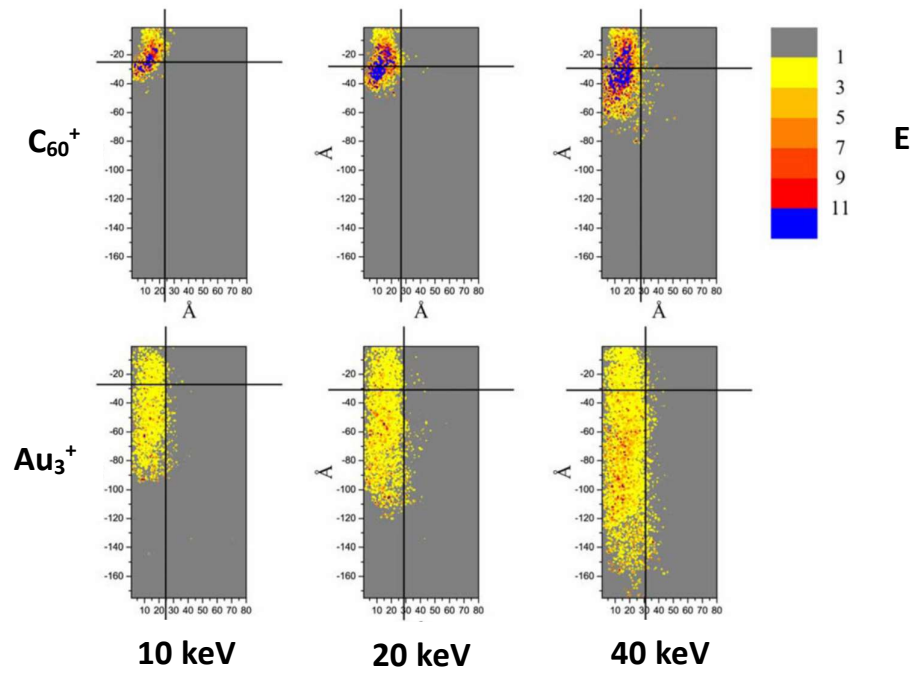


Figure 9 – Plots of the excitation energy E for C_{60} and Au_3 bombardment of ice films.

10, 20, and 40 keV ion beam energies are shown, the colour represents excitation energy, E in a contour plot

1.7.3. GAS CLUSTER ION BEAM FOR BIOMATERIAL ANALYSIS

SIMS depth profiling of organic solids and polymer films is frequently performed.

If such profiling were able to access both elemental and molecular depth profiles, this would greatly enhance the range of this technique.

Ion induced damage of organic sample surfaces unfortunately leads to a loss of molecular specificity within the depth profile layers, however we now move toward cluster ion primary beams, opening up the possibility of profiling organic materials. Multilayer organic materials were first depth profiled with the use of a SF_5^+ as an etch tool by Wagner et al⁹⁶. Furthering this, Szakal et al⁹⁷ used these cluster ions to depth profile poly(methyl methacrylate) (PMMA) films on silicon. Norrman et al also employed SF_5^+ sputter depth profiling to investigate PMMA and poly(vinyl chloride) (PVC) films spin coated onto a silicon wafer.

Following the isolation of buckminsterfullerene in 1985 by Harold Kroto⁹⁸, numerous studies have been performed to characterise the molecule. Assessment of the varying ionisation energies of C_{60}^+ by the controlled vaporisation of powders in vacuum was performed by Scheider et al. The first practical experiment using C_{60} primary ions guns for ToF-SIMS was performed by Wang et al., demonstrating that the electron impact energy in the source could be varied by filtering the ion beam with a mass gate.

The Initial studies with C_{60} observed low damage cross sections compared to gallium and SF_5 unlocking the potential to proceed beyond the static limit whereupon significant damage is caused in the underlying analysed material⁹⁹. This was shown by the Winograd group, where low-energy (10 keV) C_{60}^+ bombardment of polymers was used to remove surface contaminants from

polymers before XPS analysis. C_{60}^+ as compared to smaller cluster ions such as Au_n^{n+} and Bi_n^{n+} disintegrates into its constituent atoms as it strikes the surface. The energy of the cluster is limited to the surface allowing molecular information to be maintained as a function of its primary ion dose, whereas use of a smaller projectile causes penetration into the sample and deposition of energy into many layers below the surface. This results in subsurface mixing, causing damage deep into the sample.

C_{60} is widely considered a very effective beam of molecular depth profiling, C_{60} depth profiling of polypeptide film in trehalose⁹⁹ and organic delta layers¹⁰⁰ (**Figure 10**) are just some examples of successful studies. However, analysis of certain polymers is still proving to be difficult. Impact of C_{60} on certain polymers may catalyse cross linking as the energy is dissipated in the surface region, other effects such as rapid loss of the secondary ion signal and a decrease in sputter yields are also encountered. For hydrogel analysis this is problematic, as the macromolecular chain structure of hydrogels may catalyse cross-linking with C_{60} use. Methods of reducing damage involve sample cooling, varying the incident angle and the energy of the primary ion beam.

Argon gas cluster ion beams (Ar-GCIBs) have shown promising results in the depth-profiling of organic/biological material, particularly for samples that have proved difficult under C_{60} bombardment.

Kirkpatrick and co-workers first explored the application of gas cluster ion beams (GCIBS) in SIMS¹⁰¹, originally developed for sample cleaning and polishing in the semiconductor industry. The group reported the usage of ion beams, generated

with an average cluster size of 2000 atoms. Early simulation work around these beams proposed potential benefits in their usage, such as high sputter yields, higher secondary ions signals and minimal development of ion beam associated topography and chemical damage over C_{60} . Shard et al¹⁰⁰ reported a stable Al signal through depth profiling of organic semiconductor materials with argon clusters observing that the erosion of the sample is linearly dependent on the primary ion fluence, as a contrast to the variable signal when using C_{60} .

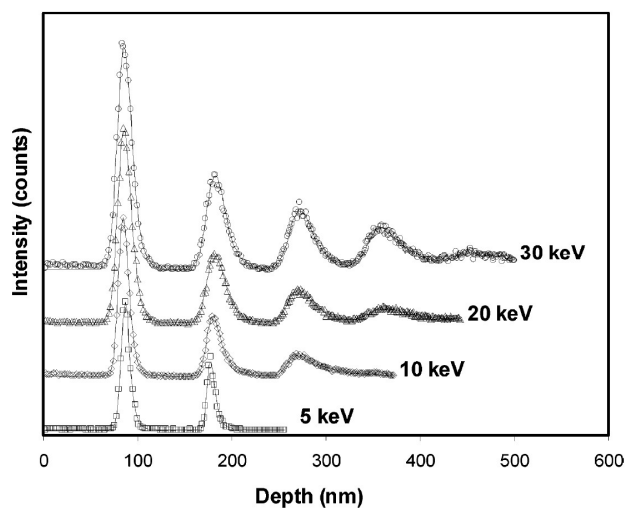


Figure 10 – Molecular depth profiling of Irganox 3114 delta layers with C_{60} .

Varying C_{60} ion beam energies are shown through the depth profile.

Shard, A et al. *Physical Chem.* 2008, 2596–2605.

1.7.4. TOF-SIMS OF HYDRATED MATERIALS

The ultra-high vacuum technique of ToF-SIMS is challenging when it comes to the analysis of hydrogels, as the low pressure reduces the vapor pressure of water promoting evaporation^{21,102} which results in structure deformation¹⁰³. Formidable challenges are present in sample preparation to ensure hydration of the material is maintained when analyzed.

Analysis of cells and tissue in a frozen state or “cryofixation” in the field of electron microscopy has been applied since the 1950’s, developing sample preparation methods to ensure the sample remains in a viable frozen state. In order to obtain meaningful results about biological cells *in vivo*, it is paramount that sample preparation does not induce significant physical changes. ToF-SIMS has the added requirement, however, that the preparation procedure also does not induce significant chemical changes.

The method of sample preparation is vastly important in SIMS analysis of materials containing water. Typically the sample, be it cell, tissue or gel contains an undetermined volume of water, and is sensitive to changes in pressure and temperature. This is problematic for ToF-SIMS as the vacuum environment required for analysis effectively dehydrates the hydrogel in ambient conditions resulting in shrinkage and redistribution of sample components.

The differences in structure of hydrogels in hydrated and dehydrated state can be seen in **Figure 11** where Al-Abboodi *et al.* characterised the open pore morphology of gelatin hydrogels by focused ion beam (FIB) microanalysis¹⁰⁴.

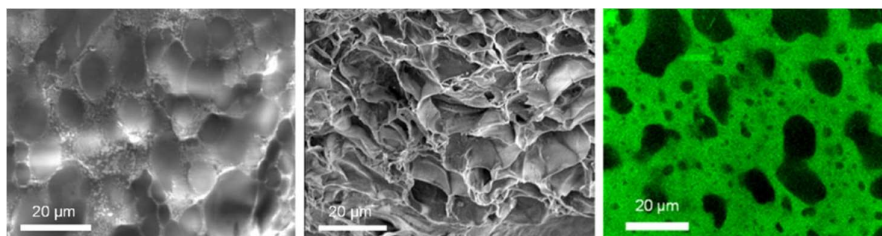


Figure 11 – Gelatin-hydroxyphenylpropionic (Gtn-HPA) hydrogels in hydrated or dehydrated state.

a) ESEM image of hydrogel surface in hydrated state, b) ESEM image after freeze-drying, c) LSCM of hydrogel with FITC labelling.

Vacuum effects on tissues have been well established¹⁰⁵. The reduced pressure around the sample translates to a reduction in water boiling point, increasing drying rate. Vacuum drying performed at 29.99 – 59.78 mbar results in a lowered boiling point of around 70°C. This has been well documented in literature in dehydrated foodstuffs, through a simple comparison of ambient versus low vacuum effects¹⁰⁶.

Solving this problem requires changing the sample state to resist vacuum induced dehydration. This could be achieved through analysing in a cryogenic state by freezing the sample, and maintaining this whilst performing analysis as the sample state is altered from a liquid to a solid.

Sublimation of ice however must be considered. In cell or tissue samples ice sublimation under vacuum may cause shrinkage and cracking redistribution cellular components, which may be experienced in freeze-dried preparation^{107–109}.

The rate of ice sublimation under vacuum is strongly affected by the sample temperature. Kossacki et al¹¹⁰ demonstrated this on demineralised and mineralised ice films, showing sublimation dramatically increased above 200 K at 5×10^{-5} mbar, other examples in literature are also reported^{102,111,112}.

The sample must therefore remain below this temperature, ideally as low as possible in order to minimise ice sublimation. This involves 'snap' freezing the sample via a cryogenic liquid, rapidly inducing formation of amorphous ice in the internal structure of the hydrogel or cell and maintaining a constant volume of water.

This prevents ice formation which may damage and disrupt the material composition. A recent publication identified a novel Gelatin self-signalling matrix where freeze fracture of the gel was observed by confocal microscopy upon cooling the gelatin dip coated film in a freezer¹¹³.

The preparation of a frozen hydrated sample for analysis in a vacuum environment is however far from trivial, it must be suitable to both retain water in the vacuum environment and limit ice formation on the sample surface. Maintaining sample temperature below -120°C is therefore paramount to reduce sublimation induced damage¹¹⁰.

The challenges in hydrated measurement begin with the method of freezing and the sample preparation employed. The cryogen used to plunge freeze the sample must be sufficiently dense to disrupt the Leidenfrost effect that occurs when a sample is warmer than the immersion cryogen causing insulation. Liquid ethane has a density of 808.607 kg/m^3 as opposed to that of liquid nitrogen (1.36 kg/m^3), therefore is preferential for rapid freezing. Sample transfer from the cryogen to the entry chamber followed by loading into the entry chamber then must be considered, as minimising the transfer time from the cryogen to the sample entry chamber is of paramount importance to reduce frosting of the

surface which would affect the quality of the data acquired as frosting can generate sputter induced topography.

The entry/analysis chamber cooling method must also be efficient enough to maintain the sample temperature below 200 Kelvin¹¹⁴, to maintain internal vitreous ice within the sample, rather than hexagonal crystalline ice formation (sample fracture).

The frozen-hydrated format is however not without its benefits. Increased secondary ion yields may be observed, a result of proton donation from incorporated water. Frozen hydrated, and dehydrated ToF-SIMS analysis of a series of seven poly (allyl methacrylate-g-dimethyl siloxane), AMA-g-DMS, graft copolymers has been reported¹¹⁵, where changes in surface chemistry was observed between dehydrated and hydrated state.

Formation of high mass oligomeric ion distributions for systems with PDMS bulk content were detected at 15 wt% in the dehydrated hydrogel, compared to 25% in the hydrated state.

1.8. X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) also known as electron spectroscopy for chemical analysis (ESCA) is one of the most widely used surface analysis techniques¹¹⁶. XPS may be employed to measure the top 0-10 nm elemental composition of the surface, to give valuable quantitative information on elemental amounts and chemical state of the surface of the material being studied¹¹⁷. Information is gained by irradiating the material with a beam of X-rays which generates photoelectrons allowing the measurement of the kinetic energy and number of electrons that escape from the material.

A schematic is shown in **Figure 12** detailing the photoelectric effect that allows XPS measurement of materials. The binding energy (E_b) is related to the energy of the incident x-ray beam ($h\nu$), subtracted by the kinetic energy of the measured ejected photoelectron E_{kin} minus the work function W_f .

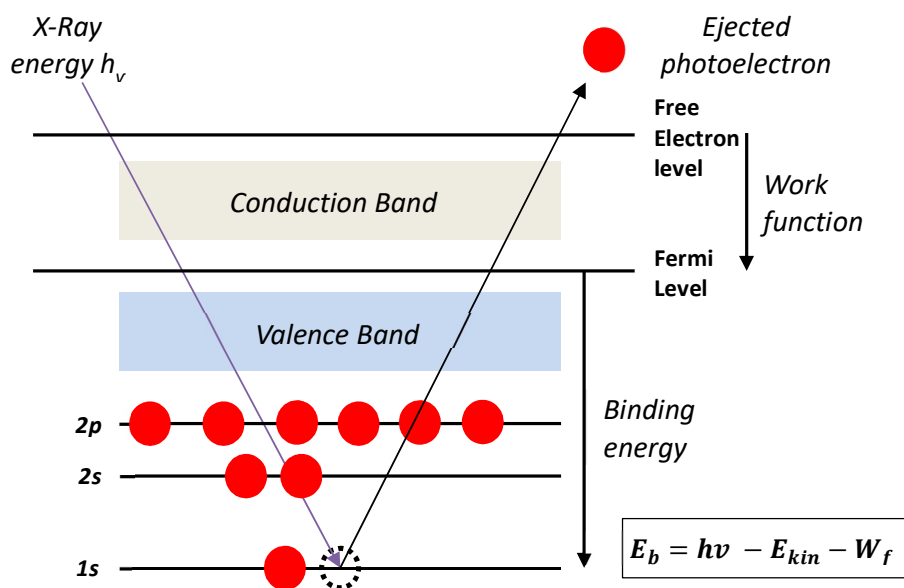


Figure 12 - The interaction of x-rays on core shell electrons

Similarly to SIMS, XPS requires a high vacuum or ultra-high vacuum to prevent attenuation of ejected photoelectrons by background gasses in the ambient atmosphere, or in the case of SIMS secondary ions.

Each element in the resultant XPS spectra provides a characteristic set of peaks at characteristic binding energy values that directly identify each element that exists in or on the surface of the material being analysed.

An example is given below in **Figure 13**, where analysis of a copolymer dip coated film is shown along with uncoated glass. The number of detected electrons in each of the characteristic peaks is directly related to the atomic concentration of each element, which allows the calculation of the concentration of each element as shown in **Table 4**.

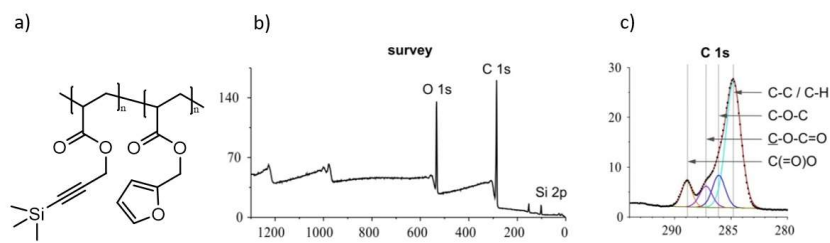


Figure 13 - XPS analysis of copolymer dip coated film

a) Chemical structure of 3-(trimethylsilyl) propargyl methacrylate-fan-furfuryl methacrylate copolymer. b) Resultant survey scan from XPS analysis of copolymer dip coated film. c) High resolution C1s spectrum.

Table 4 - Elemental composition of 3-(trimethylsilyl) propargyl methacrylate-fan-furfuryl methacrylate (p1) and control glass surfaces (glass)

Surface	% C	% O	% Si	% Sum of Rest
glass	13.6 ± 0.9	51.9 ± 0.5	22.7 ± 0.2	11.8 ± 0.5
P1	73.9 ± 0.9	20.2 ± 0.6	5.6 ± 0.4	< 1.0

While ToF-SIMS, when compared to XPS, provides the ability to uniquely identify molecular fragments, it does not readily allow for quantitative measurement. Semi-quantitative assessments of surface densities have been used in literature but these are associated with considerable uncertainties and do not replace the need for reliable quantification¹¹⁸. XPS has emerged as a complementary surface analysis technique to SIMS, as detailed molecular structure information may be collected along with quantitative elemental concentrations. For example XPS and ToF-SIMS are most frequently used to gain information on the type and amount of amino acids/peptides present on bifunctional surfaces¹¹⁹.

The sampling depth of XPS is limited to 10nm, beyond which accessing the underlying material requires removal of the upper 10nm. Ion bombardment may be employed similarly to dual beam ToF-SIMS to remove material in the spectrometer. The experiment begins with an analysis of the undisturbed surface using either XPS or ES, collecting a surface spectrum. Following this, etching similarly to ToF-SIMS depth profiling is applied to the sample surface by an inert gas, such as argon. Irradiation by the X-ray source and analysis is cycled between etchings with the sputter beam.

When collecting the spectroscopic data from the crater it is important to limit the data acquisition to the appropriate area within the crater, avoiding the area close to the walls where the crater bottom is not flat. Elemental concentrations may then be determined as a function of depth in a profile, an example of which is given below in *Figure 14*.

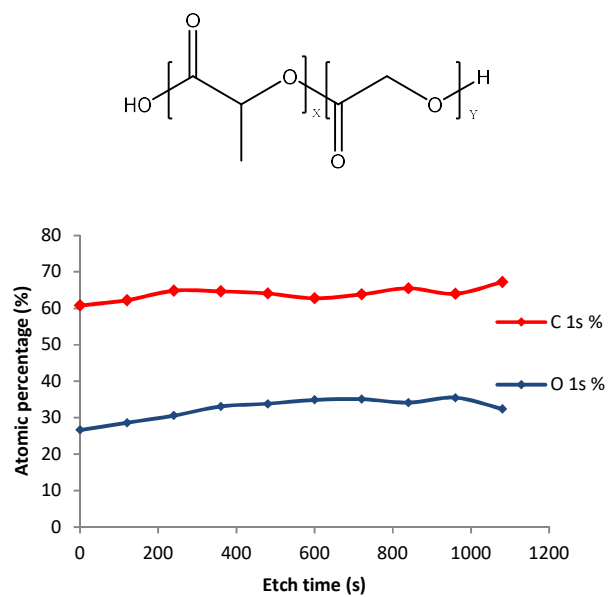


Figure 14 - XPS Ar₁₄₅₅ 2.5 KeV depth profile of poly(lactic-co-glycolic acid) film

The chemical structure of PLGA is shown above the chemical depth profile of the dip coated PLGA film, carbon is shown in red and oxygen in blue

1.8.1. QUANTITATIVE TOF-SIMS ANALYSIS

The information provided through ToF-SIMS analyses includes the nature of the surface species, chain scissions and branching's. However, its use as a quantitative tool for surface analysis is limited due to complexity in fragmentation, reorganization reactions and matrix effects.

XPS and ToF-SIMS may be applied in tandem to apply quantitative elemental amounts to the molecular information acquired by ToF-SIMS. This may be applied to static analysis of the surface composition in 3D (**Figure 16**) acquiring quantitative molecular information as a function of depth¹²⁰. This may be demonstrated by Shard et al where layered samples with known binary compositions of Irganox 1010 and Fmoc-pentafluoro-L-phenylalanine (PFLPA) were depth profiled by XPS and ToF-SIMS, to probe if matrix effects were present in organic multi-layered samples. **Figure 16c** displays both XPS and ToF-SIMS depth profiles of this sample with the relationship between volume fraction of PFLPA and normalised intensity of marker attributed to PFLPA, indicating that the secondary ion intensity of $m/z = 953$ secondary ion does not follow a linear trend with PFLPA volume fraction¹²¹.

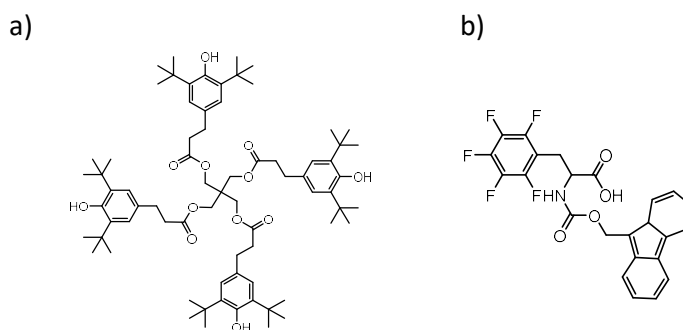


Figure 15 – Chemical structure of Irganox 1010 (a) and PFLPA (b)

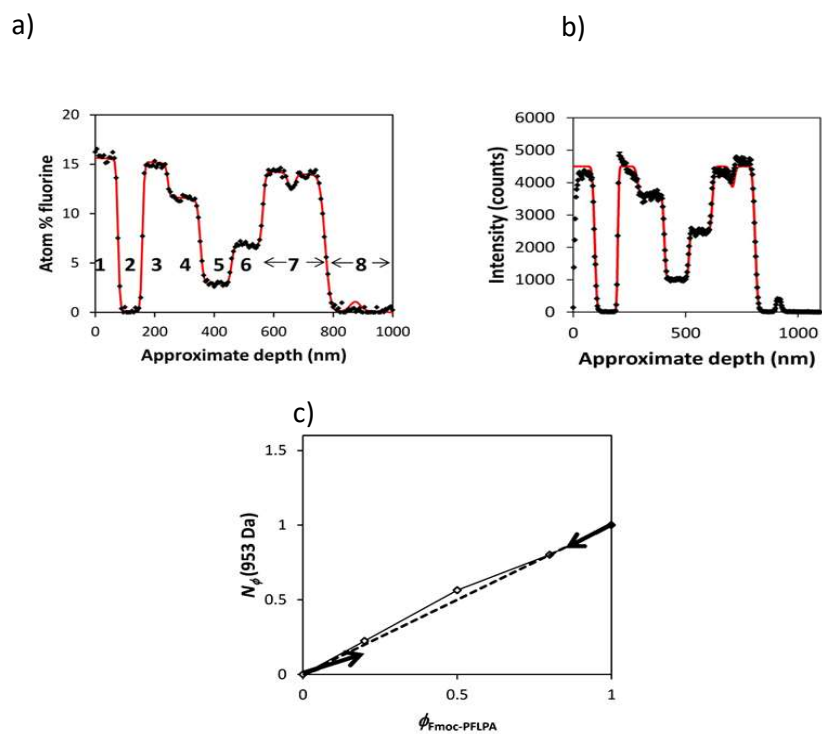


Figure 16 - ToF-SIMS and XPS of a multilayer film

a) XPS depth profile, b) ToF-SIMS depth profile, c) Measuring change in normalised intensity against volume fraction of PFLPA

Shard et al. *J. Phys. Chem. B* 2015, 119 (33), 10784–10797.

RESEARCH CHALLENGE OBJECTIVE

In order to understand the cell response to hydrogels, which is guided by adsorbed biomolecules and underlying material, characterisation of this complex material is necessary. The impact of surface chemistry of hydrogels on surface or 3D supported cells may be dramatic, however the ability to quantitatively examine the molecular composition of hydrogels in a 3D hydrated format that represents their hydration state in culture conditions is still absent. We aim here to demonstrate the first example of the label free characterisation of a hydrogel by ToF-SIMS and XPS in a frozen hydrated format, reflecting characterisation in a format most reflecting its native hydrated state. We aim to map the mobility of biomolecules in a label free manner in 3D, furthermore allowing quantitative determination of biomolecule concentrations in hydrogels.

CHAPTER 2 - MATERIALS AND METHODS

Materials. Poly(2-hydroxyethylmethacrylate) (1% wt/v, $M_w \sim 20,000$, Sigma Aldrich) was dissolved in ethanol by sonication overnight at 20°C. A silicon wafer substrate (5 × 5 mm) was prepared by first sonicating with water, acetone and lastly ethanol. The wafer was dip coated in the pHEMA solution at a retraction speed of 2 mm/s three times and allowed to dry for 1 hour. 1-3 coats of the 1% pHEMA solution were applied and the samples left to dry. pHEMA hydrogel films were prepared by dissolving Poly(2-hydroxyethylmethacrylate) (6% wt/v, $M_n \sim 20,000$, Sigma Aldrich) in ethanol by sonication overnight at 20°C. A silicon wafer substrate (5 × 5 mm) was prepared by first sonicating with water, acetone and lastly ethanol. The wafer was dip coated in the pHEMA solution at a retraction speed of 2 mm/s three times and allowed to dry for 1 hour. To investigate the ingress of protein into the films, the polymer dip coated wafer was immersed in a lysozyme (1% wt/v, Sigma Aldrich)/Milli-Q grade deionised water solution at room temperature for 16 hours. A pHEMA (6% wt/v) and lysozyme (1%wt/v) ethanol solution was also prepared and dip coated onto silicon wafers. Similarly the silicon wafers were immersed in deionised water for 16 hours. Ellipsometric measurements of the films before analysis were performed using an Alpha-SE ellipsometry with completeEase software for modelling in fast mode over a 380-900 nm wavelength range at 45° beam angle. Measurement of film thickness of the pHEMA/lysozyme hydrogels was attempted using a liquid cell however due to the opaque nature of the films, assessment of thickness was not possible.

Swelling study. A mass balance sensitive to 5 decimal places was used to determine the average mass of silicon wafers for the swelling study (n=12) before

the pHEMA 1-3 coatings were applied. pHEMA films were then incubated for 12 hours in deionised water at 20°C, then weighed again to determine the mass difference between hydrated and non-hydrated.

ToF-SIMS Frozen Hydrated Sample Preparation. For ToF-SIMS analysis, hydrated films were mounted onto an ION TOF cryo-stage, one sample at a time (*of dimensions 5 × 5 mm*) followed by plunging into liquid nitrogen for 5 minutes. The sample stage was removed from the liquid nitrogen and placed onto the pre-cooled sample transfer arm in the entry chamber under nitrogen flow ($T \sim -70^{\circ}\text{C}$). Prior to entry of the hydrated films to the ToF-SIMS entry chamber, the sample surface was evacuated with a high flow of argon gas to remove ice (frost) formed on the sample stage in an 'air gun' fashion. Upon reaching a pressure of 2×10^{-6} mbar the entry door to the main chamber was opened and the cryo-stage introduced for analysis. Sample temperature did not exceed -70°C in the entry chamber, maintaining at -110°C under a 2×10^{-6} mbar vacuum. Analysis was performed at -120°C in the main chamber, the sample was maintained below this temperature when depth profiled to reduce the possibility of surface ice crystallization.

Instrumentation. ToF-SIMS data were collected using a ToF-SIMS IV instrument (Münster, Germany) equipped with a bismuth liquid metal ion gun and argon cluster sputter gun. The analysis beam for this study was generated by the liquid metal ion gun, specifically a 25 keV bismuth source, utilizing a Bi_3^{++} rastered over an area of $100 \times 100 \mu\text{m}$ with 128×128 pixels. The target current was measured as 0.3 pA with a total primary ion dose of 9.2×10^{10} ions/cm². A 10 keV Ar1455

cluster ion source was employed to etch through the sample over a 400 x 400 μm area. Rastering was performed in a non-interlaced mode with 1 frame of analysis and 3 s for sputtering per cycle. The corresponding beam dose was determined to be 6.84×10^{12} ions/ cm^2 . An argon beam target current of 9 nA was employed for all samples analysed allowing comparison of sample thicknesses from time taken to reach the hydrogel-silicon wafer interface. Postprofiling crater analysis was attempted with the aim of determining the crater depth for each sample, however, on warming the films to room temperature topography developed, likely indicating delamination of the films, preventing accurate film thickness analysis. A low energy electron flood gun was employed for charge neutralization. Data processing was done with the commercial IONTOF software, SURFACELAB6. Secondary ion assignments were selected by referring to a reference database of secondary ions related to the components of the material analysed.

XPS Frozen Hydrated Sample Preparation. Samples of PLGA on glass coverslips were provided by Dr Emily Smith. For XPS analysis, hydrated films were mounted onto an XPS sample stub and pinned with a copper bar, one sample at a time (*of dimensions 5 x 5 mm*) followed by plunging into liquid nitrogen for 5 minutes. The stub was removed from the liquid nitrogen and placed onto the pre-cooled sample transfer arm in the entry chamber under nitrogen flow ($T \sim -70^\circ\text{C}$). Upon reaching a pressure of 5×10^{-9} Torr the entry door to the main chamber was opened and the stub introduced for analysis. The sample did not exceed a temperature of -70°C in the entry chamber, maintaining -160°C under a 5×10^{-9} Torr vacuum. Analysis was performed at -160°C in the main chamber, the sample was

maintained below this temperature when depth profiled to reduce the possibility of surface ice crystallization.

Instrumentation. The pHEMA hydrogel films were analyzed in a Kratos Axis Ultra XPS with a mono-chromated Al $k\alpha$ X-ray source (1486.6eV) operated at 120 W operated in FoV1, 110 μm aperture mode, and a Argon₁₂₀₀ 10 Kev sputter source operated at 0.3 pA. Cycling between etching and analysis was performed by 30 seconds analysis, then 120 seconds of etching with the argon cluster sources. High resolution peaks were acquired in standard spectroscopy mode with a step of 0.1 eV PE 40 for 30 s each. The charge neutralizer filament (CN) was used to prevent sample charging during analysis. Cycling between The analysis chamber pressure was better than 5×10^{-9} Torr prior to analysis, but due to the argon flux from the GCIS the pressure increased to 2×10^{-8} Torr. Data processing was performed using CASAXPS™ (version 2.3.17DEV6.6h) with Kratos sensitivity factors (RSFs) to determine atomic percentage values from the peak areas. Spectra were not charge corrected for this work.

Stability of activated surfaces under ToF-SIMS/XPS. For ToF-SIMS analysis, frozen hydrated samples were prepared in the same manner to the previous entry reported, and profiling to the interface between ice and pHEMA was performed, upon which an area $100 \times 100 \mu\text{m}$ with a 128×128 pixel density. For XPS analysis, hydrated films were mounted onto an XPS sample stub and pinned with a copper bar, one sample at a time (*of dimensions $5 \times 5 \text{ mm}$*) followed by plunging into liquid nitrogen for 5 minutes. The stub was removed from the liquid nitrogen and placed onto the pre-cooled sample transfer arm in the entry chamber under nitrogen flow

($T \sim -70^{\circ}\text{C}$). Upon reaching a pressure of 5×10^{-9} Torr the entry door to the main chamber was opened and the stub introduced for analysis. Sample temperature did not exceed -70°C in the entry chamber, maintaining at -160°C under a 5×10^{-9} Torr vacuum. Analysis was performed at -160°C in the main chamber, the sample was maintained below this temperature when depth profiled. Sputter depth profiling was performed until the interface between ice and pHEMA was reached, upon which the sample was irradiated with mono-chromated Al $K\alpha$ X-ray source or Bi_3^{++} primary ion beam in the case of ToF-SIMS.

CHAPTER 3 - AR-GCIB DEPTH PROFILING OF & DETERMINING PHEMA HYDRATION CHARACTERISTICS

3.1. AIM

In this study we describe the surface and bulk chemical analysis of pHEMA.

This was performed to study the behaviour of pHEMA dip coated films under Argon sputter ToF-SIMS depth profiling. C_{60} is widely employed as a sputter etching beam for polymer analysis, however it is well known to generate damage when irradiating polymers including cross-linking and variable erosion rates. We show that Argon clusters do not induce chemical damage in the pHEMA film, evidenced by stable $C_6H_9O_2^+$ Ion Intensity, and depth profiling the films of different thicknesses can be performed. In this study we also compare the swelling properties of pHEMA films with the aim of determining the optimum pHEMA film thickness for a viable sample that may be chemically characterised through ToF-SIMS in a hydrated state.

3.2. INTRODUCTION

Poly(2-hydroxyethyl methacrylate) (pHEMA) is a polymer that forms a hydrogel. It was first applied in intraocular lenses, synthesised by solution polymerisation using 2-hydroxyethyl methacrylate (HEMA). Drahoslav Lim and Otto Winchterele first isolated and patented the material in 1953, where they succeeded in preparing a cross-linking gel which absorbed 40 times its mass in water³. Winchterele identified the isolated polymer as “a structure permitting the desired water content, and inertness to normal biological processes including resistance to the degradation of the polymer and to reactions unfavourable to the organism”³. Most importantly he observed that produced polymer films allow for permeability of metabolites, the importance of which will be discussed later. pHEMA scaffolds present an open-pore morphology, in which the pores are larger than 10 μm in diameter and uniform throughout the material allowing for cellular proliferation and tissue development^{122–124}. The biocompatibility of pHEMA and its permeable nature has resulted in the widespread use of the material in bioengineering, a number of which include (*Figure 17a-d*): artificial cornea¹²⁵, artificial skin¹²⁶, drug delivery systems¹²⁷ where covalently attached to peptides, bone composite materials and tissue support scaffolds¹²⁸.

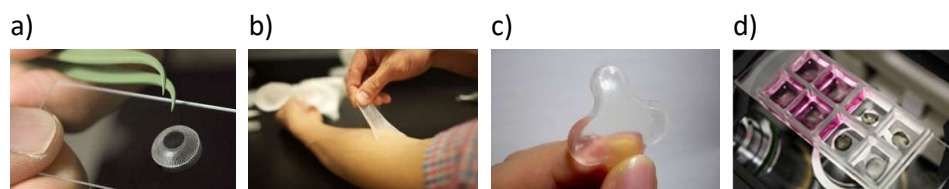


Figure 17 – uses of pHEMA hydrogels in bioengineering.

a) artificial corneal replacements, b) artificial skin, c) bone composite materials, d) tissue support scaffolds

Recently argon gas cluster ion beams have become available to allow for depth profiling of organic materials with minimal chemical degradation, constant sputtering yields, and good depth resolutions¹²⁹. C₆₀ as a sputtering source may result in cross-linking of certain polymers during the sputtering process^{94,130}. This results in a decrease in ability to sputter through the polymer, the outcome of which is an unmeasurable sample. Various modifications to the experimental setup have been developed in an attempt to overcome this problem. This includes sample cooling, sample rotation, the use of grazing angles of incidence etching beam and nitric oxide gas dosing¹³¹, which have all exhibited varying degrees of success.

The most comprehensive study of sample damage has been demonstrated by Nieuwjaer et al⁹⁵ in depth profiling of polystyrene (PS) thin films using C₆₀⁺. They observed for a 794 molecular weight polystyrene film, an exponential decrease of the signal corresponding to PS. The ion signal representative of PS (C₇H₇⁺) is lost before the interface region between PS and glass substrate is reached corresponding to a signal rise for the silicon substrate marker (*Figure 18*) 1×10^{14} ions/cm². This indicated that depth profiling for this material composition with C₆₀⁺ was not possible.

Before developing a method for analysis of pHEMA in a hydrated state, characterisation of pHEMA dip coated films in a dehydrated state was performed to ensure that the sample preparation was suitable to develop flat surfaces with minimal topography. Furthermore determine that Argon is a suitable etching beam for chemical analysis of a dip coated pHEMA films.

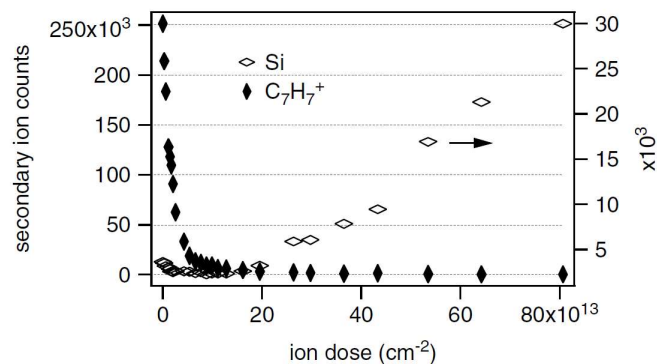


Figure 18 - Sample damage in C_{60} Depth profiling a poly(styrene) film

Depth profiling a 794 M_w poly(styrene) thin film spin coated on a silicon wafer with C_{60}^+ primary ions. Si^+ indicated by the white diamond marker is attributed to the silicon wafer substrate, whilst $C_7H_7^+$ is attributed to the poly(styrene) film.

N. Nieuwjaer, C. Poleunis, A. Delcorte, and P. Bertrand, *Surf. Interface Anal.*, 2009,

Polymer hydrogels swell, but do not dissolve when water is added. The degree of swelling depends on many factors such as network density, solvent nature and polymer solvent interaction. The properties of water swelling of pHEMA hydrogel films were studied in this work, to determine the water content and swelling capacity of dip coated pHEMA as this would allow us to determine the appropriate amount of incubation time to ensure a hydrated film whilst preventing delamination from the silicon wafer substrate which may be experienced when swelling physisorbed polymer films on flat substrates¹³².

3.3. RESULTS

In chemically profiling pHEMA, we first wanted to validate the secondary ion fragments reported in the literature for pHEMA¹³³, establish a method to produce uniform pHEMA films, determine the depth resolution by Ar-GCIB sputter depth profiling and calibrate etch time to thickness, prior to swelling the pHEMA gel and performing the measurement in a frozen-hydrated state. This would allow us to determine the degree of gel swelling in pHEMA films to understand the swelling behaviour of pHEMA films. The swelling behaviour of pHEMA has been well documented as they are frequently used in ophthalmology^{66,81}. However determining the effect of swelling on the viability of the physical sample state was important to assess if delamination or surface defects were occurring as a result of gel swelling. Initial assessment of the surface of dip coated films revealed that the surface topography was within 5 nm (*Figure 19*). 25 KeV Bi₃⁺⁺ ToF-SIMS in the positive mode of the 1 wt% pHEMA three times dip coated film is shown in *Figure 20* with the major secondary ion fragments and structures in *TABLE 5*. The signal intensities on the surface are high with respect to organic associated peaks including characteristic organic polymer associated ions such as C₂H₅⁺, C₃H₃⁺, C₃H₅⁺, C₂H₃O⁺ and C₂H₅O⁺. A mass fragment at m/z 113 representative of the HEMA monomer unit C₆H₉O₂⁺ was used as a marker for pHEMA. The primary ion dose was below the static limit calculated at 1.35 x 10¹² (ions/cm²). A low deviation of -20.87 ppm was observed when assigning the C₆H₉O₂⁺ structure. Previous reports surface analysis of pHEMA have used this mass peak to assign HEMA¹¹⁶. Determination of the interface was performed by selecting Si⁺ as a marker for the silicon wafer substrate as this has also been well reported as a

positive marker for silicon¹³⁴. The lateral distribution of pHEMA on the surface appears to be uniform as evidenced by the 2D ion images in *Figure 21*.

A steady state Ion Intensity for $C_6H_9O_2^+$ is observed in the depth profile of the pHEMA coated silicon wafer by 1 dip on pHEMA. The interface region is between 80 and 140 seconds in the depth profile, with the interface position (I) at 50% maximum $C_6H_9O_2^+$ signal intensity at 117 seconds (*Figure 22*). The interface (I) and its width (I_w) are usually stated in units of thickness (nm), rather than units of time (s). To convert etch time to thickness profilometry of the etch crater was performed which is shown in *Figure 23*, where the topography of the etch crater was found to be within 5 nm. The results of the profilometry of the crater post etch for the 1-3 coatings of the pHEMA are shown in *Figure 24*.

The measured thickness for 1-3 dips in pHEMA, was assessed to be 28 ± 0.47 nm, 50 ± 0.67 nm and 53 ± 0.89 nm for one, two and three coatings respectively. The number of sample repeats for this study was 10, to investigate the repeatability of dip coating for defined film thicknesses. Depth profiles of the varying thicknesses of pHEMA films in *Figure 25* show that a stable $C_6H_9O_2^+$ secondary ion intensity at a steady state is observed in each of the profiles until a decrease at the interface region. The interface widths were calculated as 15, 18 and 17 nm for the dip coated samples of 1-3 dips respectively.

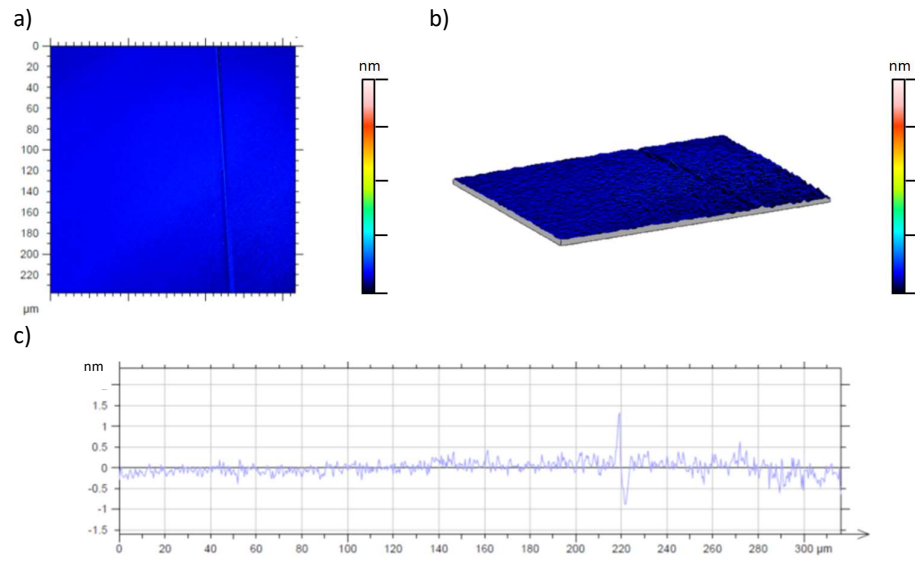
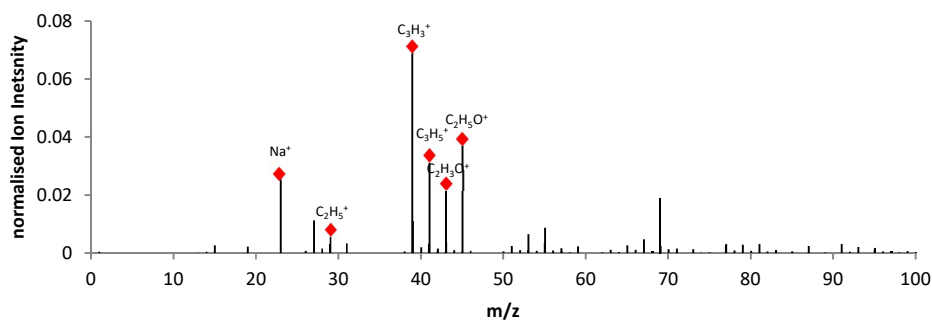


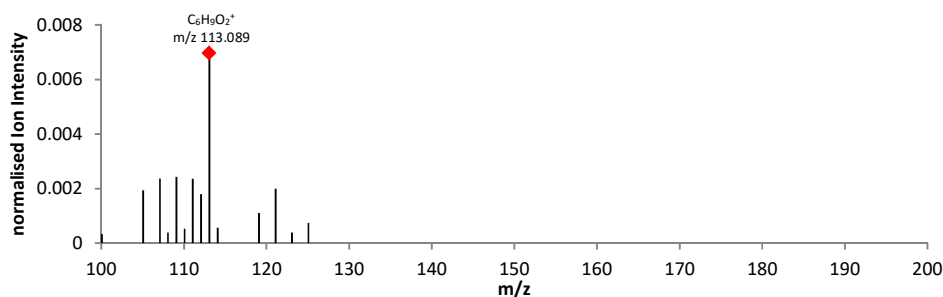
Figure 19 – Surface profilometry of pHEMA film produced by 1 dip in 1 wt% pHEMA solution.

a) 2D profile, b) 3D cross section, c) thickness extraction profile of pHEMA film

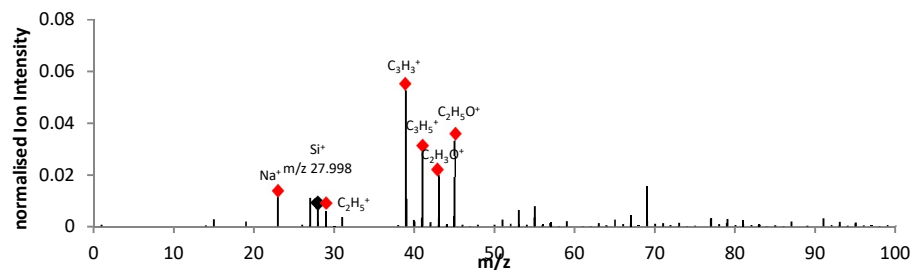
a)



b)



c)



d)

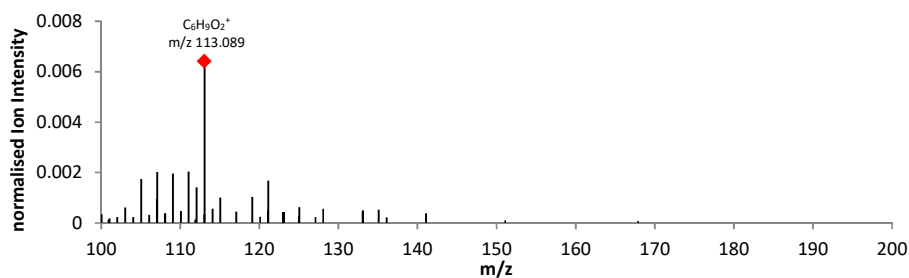


Figure 20 - ToF-SIMS spectra of pHEMA film produced by 1 dip in pHEMA/ethanol solution.

Surface spectra at a) mass range 0-100, b) mass range 100-200 m/z . Spectra at etch time of 100 seconds a) mass range 0-100, d) mass range 100-200 m/z . Ion peaks related to pHEMA and silicon are shown in red and black respectively,

$$\text{Primary ion dose} = \frac{I_p t}{A} \quad 1.35 \times 10^{12} \text{ ions/cm}^2 = \frac{(6.76 \times 10^7) \times 1}{1 \times 10^{-4}}$$

Equation 3 – Primary ion dose (ions/cm²)

I_p is primary ion of flux (ions/s), t is the analysis time (s) and A (cm²) is the bombarded surface area.

Vickerman, J. C.; Gilmore, I. S. *Surface Analysis: The Principal Techniques*; 2009.

Table 5 - positive secondary ions reported from depth profiling pHEMA film.

	m/z	Structure	Deviation (ppm)
			pHEMA
Silicon Wafer	22.999	Na ⁺	23.5
	27.034	C ₂ H ₃ ⁺	14.2
	27.987	Si ⁺	-20.1
	29.047	C ₂ H ₅ ⁺	1.4
	39.030	C ₃ H ₃ ⁺	2.7
	41.041	C ₃ H ₅ ⁺	23.9
	43.022	C ₂ H ₃ O ⁺	27.1
	45.040	C ₂ H ₅ O ⁺	42.3
	53.043	C ₄ H ₅ ⁺	21.2
	55.069	C ₄ H ₇ ⁺	31.8
	69.044	C ₄ H ₅ O ⁺	41.4
	87.051	C ₄ H ₇ O ₂ ⁺	33.2
pHEMA	113.089	C ₆ H ₉ O ₂ ⁺	54.8

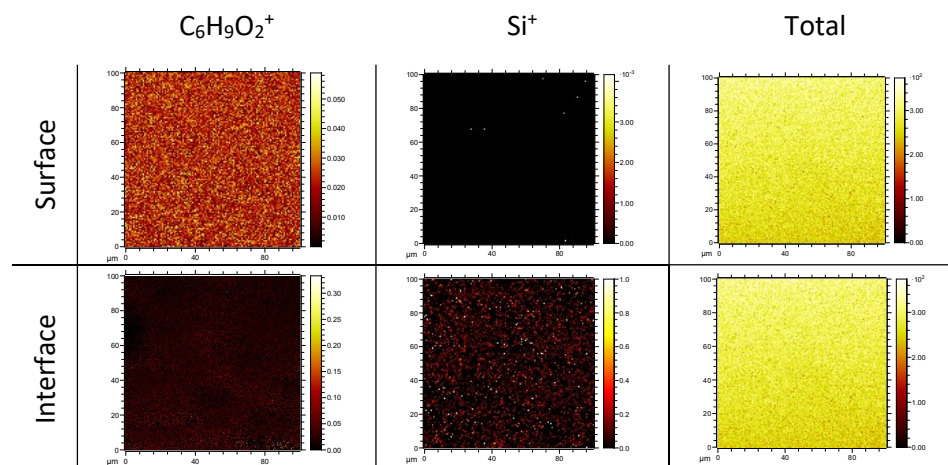


Figure 21 - 2D ion images at surface and interface regions

Retrospective lateral distribution ion images of the surface

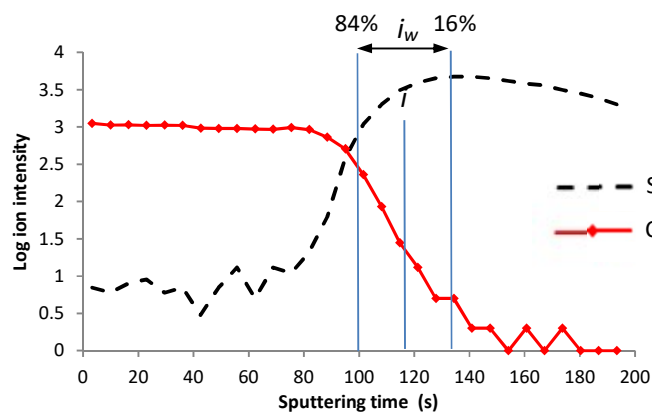


Figure 22 - Ar_{1200} ToF-SIMS depth profile of pHEMA dip coated film of 3 dips in 1 wt% pHEMA in ethanol.

Interface width is shown by I_w and interface position at I . Calculated interface based on 16-84% $C_6H_9O_2^+$ intensity as overlay

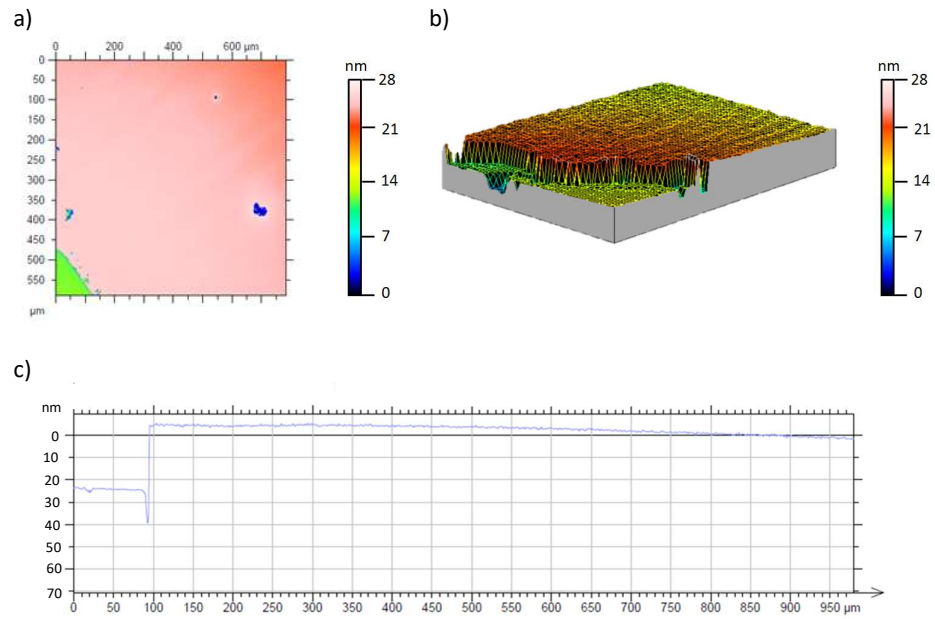


Figure 23 – Profilometry of etch crater produced by pHEMA film produced by 1 dip in 1 wt% pHEMA solution.

a) 2D profile, b) 3D cross section, c) thickness extraction profile of pHEMA film

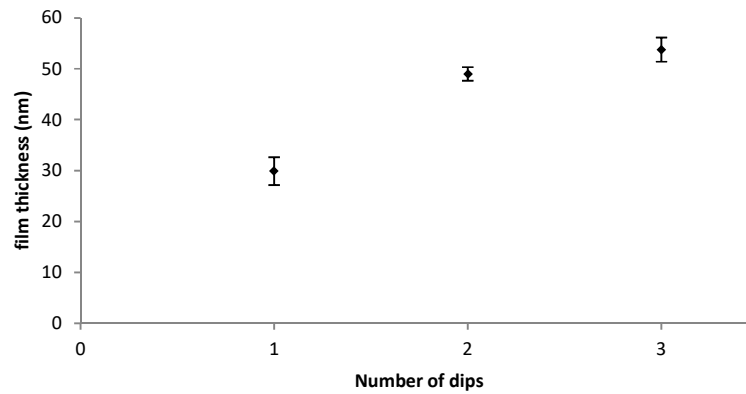


Figure 24 – Film thickness determination by profilometry from post-sputter analysis of etch crater of pHEMA film obtained after dip-coating the samples in a pHEMA solution one, two or three times.

Error bars are calculated based on standard deviation of sample size of $n=3$

Table 6 –Assigned thickness used to calculate sputter rate from 10 keV Ar₁₄₅₅ depth profiling of silicon wafer after 1-3 dips in 1wt% pHEMA

Dips (no.)	Sample Repeat (no.)			
	1	2	3	4
1	27.24	27.02	27.01	28.02
2	49.81	49.61	50.80	50.93
3	52.21	52.42	53.81	53.91

Average Thickness (nm)	Standard Deviation	C ₆ H ₉ O ₂ ⁺ intensity (50%) Interface (s)	Interface width (s)	Interface (nm)	Depth resolution (nm)	Sputter rate (nm/s)
27.31	0.47	55	20	28	8.2	0.50
50.28	0.67	101	22	50	7.8	0.50
53.08	0.90	107	21	53	8.0	0.50
Average sputter rate = 0.50 nm/s						

$$\frac{(C_6H_9O_2x_{50\%(84-16\%)})}{Thickness\ (nm)} = \Delta Z\ (depth\ resolution)\ (nm)$$

Equation 4 – depth resolution

$$\frac{\sum Sample\ thickness\ (nm)}{etch\ time\ to\ interface\ (s)} = Sputter\ rate\ nm/s$$

Equation 5 – sputter rate

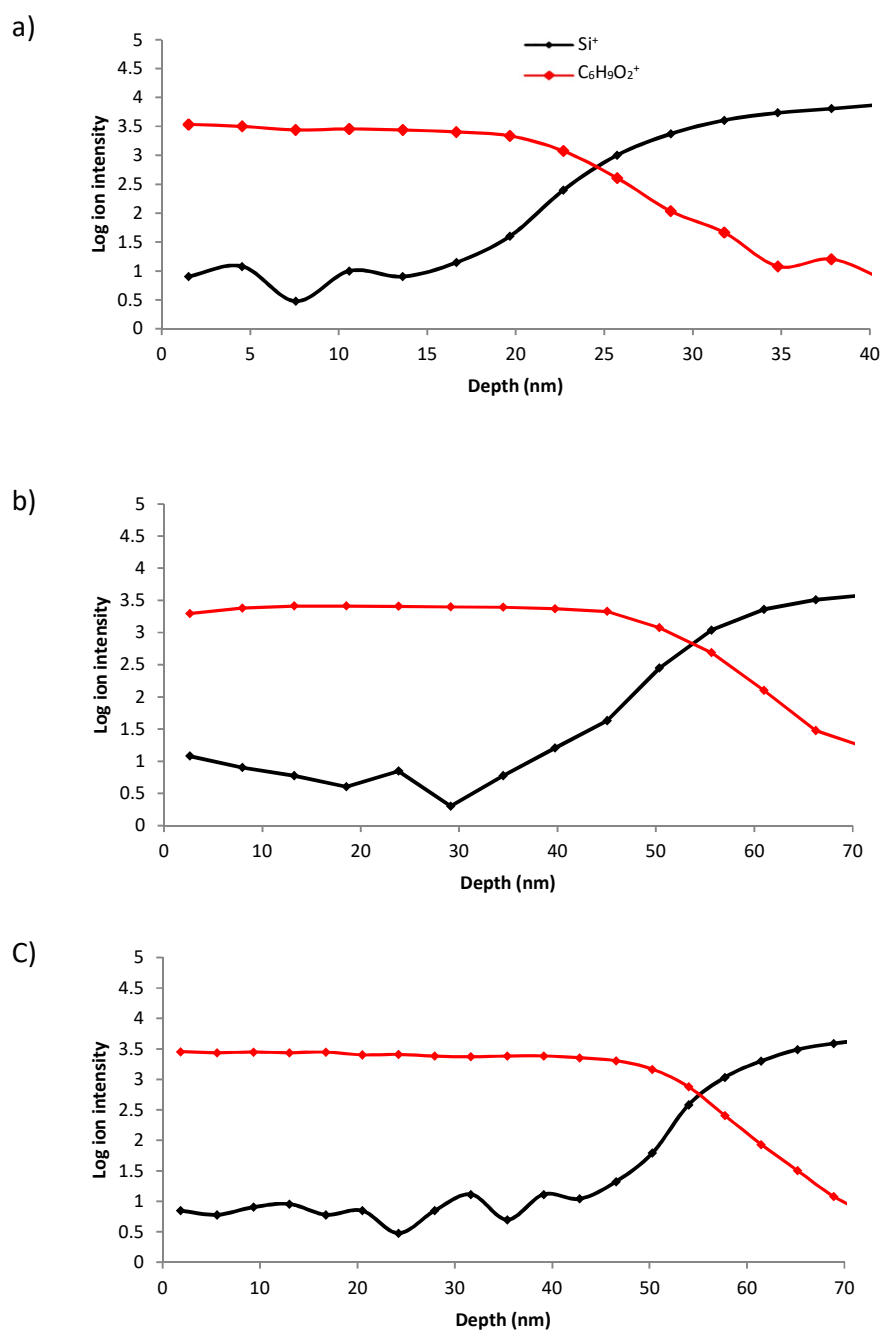


Figure 25 - Calibration of etch time to depth in nm for dip coated pHEMA films.

a) 28 nm, b) 50 nm, c) 53 nm pHEMA films. pHEMA is indicated in red by C₆H₉O₂⁺ and silicon in black by Si⁺.

The swelling ratio of pHEMA can give valuable information on mechanical characteristics of the gel. In a hydrophilic polymer network water acts as a plasticiser¹³⁵. The swelling process of the hydrogel can be considered under rubbery state and can be described by the free energy of mixing ΔG_{mix} from the polymer and solvent interaction and the elastic free energy $\Delta G_{elastic}$ from the crosslinked network⁶⁴.

$$\Delta G_{system} = \Delta G_{mix} + \Delta G_{elastic}$$

Equation 6 – Process of swelling.

Omidian, H. et al. Polym. Sci. Technol. 1994, 3 (2), 115–119.

When the film begins to swell, $\Delta G_{mix} < 0$, $\Delta G_{elastic} > 0$ and $\Delta G_{mix} + \Delta G_{elastic} < 0$, therefore swelling is favourable and the solvent diffuses into the film. Whilst swelling is occurring, ΔG_{mix} and $\Delta G_{elastic}$ both increase until $\Delta G_{mix} = \Delta G_{elastic}$. $\Delta G_{system} = \Delta G_{mix} + \Delta G_{elastic} = 0$, this means that the driving force behind swelling is lost, and equilibrium swelling has been reached. Swelling ratio is calculated by: W_s is the weight of hydrogel in swollen state, W_d the weight of hydrogel in dry state⁶⁵.

$$Swelling = \frac{W_s - W_d}{W_d}$$

Equation 7 – Swelling ratio

Omidian, H. et al. Polym. Sci. Technol. 1994, 3 (2), 115–119.

Figure 26/Figure 27 display the gravimetric measurements and the calculated swelling percentages of the pHEMA dip coated films. A higher swelling percentage is observed in the 28nm films, when compared to the 50 and 53 nm films. There is

little difference between the swelling percentage of the 50 and 53 nm dip coated films. Following the 16 hours incubation the 28 nm thinnest film showed evidence of delamination from the silicon wafer substrate.

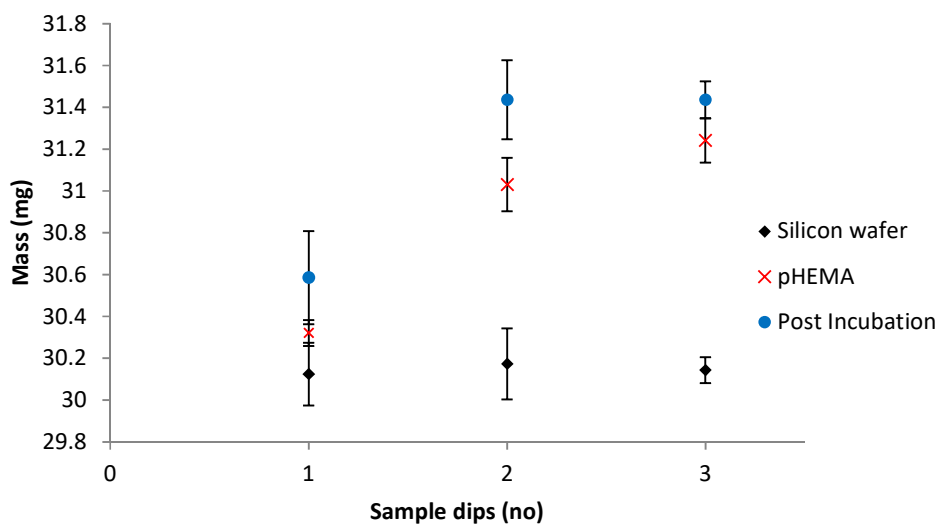


Figure 26 - Gravimetric analysis of pHEMA films in response to incubation time

Results were acquired using an analytical balance sensitive to 6 decimal places (mg) with a sample replicate of $n=6$, silicon wafer mass is shown in black, pHEMA film pre incubation shown in red and pHEMA film post incubation for 16 hours at 20°C shown in blue

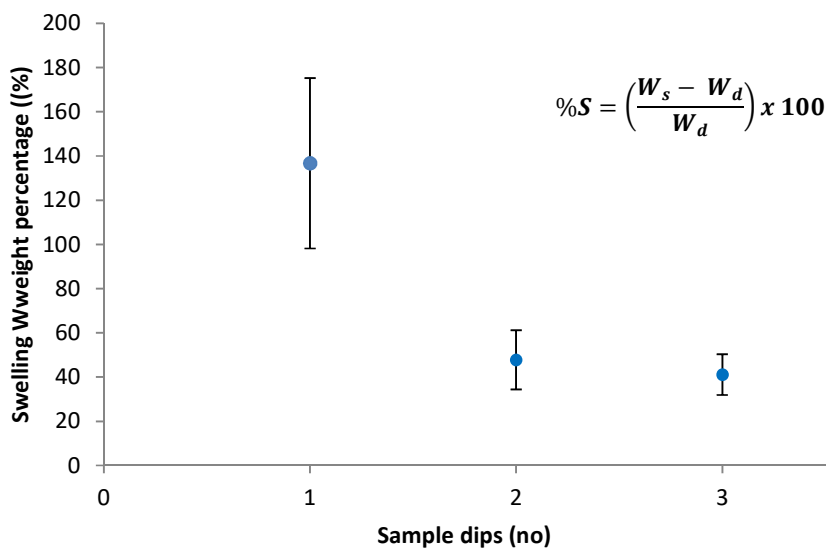


Figure 27 - Calculated swelling percentage for 1-3 dip coatings in pHEMA solution

Table 7 – Swelling percentage for 1-3 dip coats in 1wt% phema solution

Sample repeat	Swelling percentage (%) of number of dips		
	1	2	3
1	88.77	33.33	42.54
2	151.29	26.82	57.28
3	157.64	38.31	40.62
4	142.85	44.23	42.42
5	147.85	49.58	31.72
6	148.01	52.11	41.84
7	144.28	42.15	34.93
8	28.11	67.97	37.78
9	141.17	65.57	28.16
10	148.14	57.60	40.8
Average	129.82	47.77	39.76
SD	38.56	13.44	7.83

3.4. DISCUSSION

This study aimed to demonstrate the applicability of argon as a cluster source for profiling dehydrated pHEMA hydrogel films. Assessing the topography of the depth profiled sample surface is important as topography on the micron range can result in decrease in mass resolution and distortion of elemental distributions as secondary ion species are collected from differing heights in the sample. Atomic mixing induced by ion beam damage and surface roughening during sputter or analysis cycles is undesirable in depth profiling, which also may manifest from surface topography^{136,137}.

Dip coating was chosen rather than spin casting or manually coating due to previous work within the group identifying that dip coating produced the most uniform film coating as opposed to manual coating or spin casting. Analysis of the post etch crater demonstrated that the difference in feature height on the surface was within 5 nm (**Figure 23**), this is similar to the roughness range on the surface of 5nm (**Figure 19**), indicating that Argon sputtering does not induce sample roughening. This may however be the sensitivity limit of white light profilometry to detect features below this size on the sample surface, therefore 5nm may be the maximum topography measured.

There appears to be minimal difference between 2 and 3 pHEMA coats (**Figure 24**) in terms of thickness. This suggests that the ethanolic solution at for the 3rd coating results in solubilisation of the 2nd pHEMA coating, reducing the amount of pHEMA deposited on the film.

In molecular depth profiling the depth resolution is an important aspect, and is usually estimated by measuring the interface width between the organic layer and the silicon substrate. This measurement is not straightforward because the etching rate of Si is significantly lower than most organic materials. Although there is no standardized measurement approach, the most accepted way is to estimate the interface width using the 84%-16%⁹⁹. Since the depth profile data is output in the form of intensity as a function of time, this method defines the interface as the time at which the overlayer or the substrate signal intensity reaches 50% of its maximum value. In our case we used $\text{C}_6\text{H}_9\text{O}_2^+$ to qualify the interface position. The depth resolution is then calculated by measuring overlayer intensity to decrease from 84% to 16% of its maximum value. This allowed us to calibrate etch time to depth scale, which gives a more accurate description of the depth resolution as this is usually stated in nanometers rather than a unit of time. The depth resolution for each of the pHEMA film thicknesses was calculated at 8nm. It should be noted in the profiles in **Figure 25** the Si^+ signal is observed before a decrease in $\text{C}_6\text{H}_9\text{O}_2^+$ intensity, before the interface region is reached between pHEMA and silicon wafer. This may be attributed to 25 KeV Bi_3^+ analysis ejects materials from depth of up to 2 nm below the surface so seeing layers just before the interface is reached is not unexpected, and matrix effects cause the intensities not to be linearly related to quantity. This is similar to the observations seen by Shard *et al.* depth profiling Iragnox multilayers¹³⁸. The sputter rate for pHEMA was calculated to be 0.633 nm/s (+/- 0.04). Increasing film thickness therefore has no impact on depth resolution and sputter rate.

For the aim of analysing hydrogels in a hydrated state, we first had to assess the water swelling properties of pHEMA films, as this would demonstrate polymer films comprised on pHEMA had the ability to swell and hold water in the polymer network. pHEMA is well known to swell in aqueous conditions, increasing its mass percentage by up to 600%¹³⁹. Poly-HEMA functions as a hydrogel by rotating around its tertiary carbon of the methacryl group. In dry air it has been observed that, the non-polar methyl side turns to the air-film interface, making the material brittle and easy to grind into the correct lens shape. In water, the polar hydroxyethyl move to the water-film interface and the material becomes flexible. The swelling capability of pHEMA, and degree of swelling may be influenced by a number of factors including osmolality of the incubation medium and degree of cross-linking. Direct comparison between the swelling properties of pHEMA in solution and films cannot be accurately stated. Films, physisorbed to the substrate, in our case silicon wafer are held in a defined physical shape under dry conditions by interaction between the pHEMA network and the silicon substrate. Hydrogen bonding between the polar silanol groups and the free primary hydroxyl end groups in pHEMA form weak interactions adhering the pHEMA to the surface. We have observed that delamination occurs after 16 hours swelling in water for the 28nm film. Swelling deforms the physical structure of films, which suggests thin films are weakly physisorbed compared to thickener films where no delamination was observed. We determined that thicker films of 50 and 53 nm are less amenable to swelling as the swelling ratio for these thicknesses was around 90% less than the thinner 28 nm pHEMA film. Less deformation of the structure was seen in the thicker films, which is reasonable to suggest to be a factor of their

lesser ability to swell⁶⁶. This suggest the pHEMA chains in the 28nm physically cross-linked film are in a more relaxed state than the 50 and 53 nm films, while the pHEMA chains in thicker films have a level of steric hindrance that prevent the influx of water into the structure.

3.5. CONCLUSION

Analysis of pHEMA films using Ar-GCIB etching yielded some interesting results. It has been demonstrated that poly(2-hydroxyethyl methacrylate) hydrogel films produced by dip coating can be successfully chemically characterised by utilising 10 KeV Ar_{1455}^{+} cluster sputtering by ToF-SIMS.

Dip coating was found to produce films with no significant difference in feature height in the analysis area of the sample. The sample thickness may be varied by dip coating silicon wafers with a pHEMA solution, however after the 3rd dip in the pHEMA solution re-solubilisation of the pHEMA coat on the film is observed. Thinner films were observed to have greater swelling capability than the thicker films, absorbing more water, due to the more sterically hindered organisation of pHEMA chains in the thicker film, as compared to a relaxed state in the thinner films. The swelling for thicker pHEMA films is far less than for the swelling for thinner films, however delamination from the substrate occurs with thinner pHEMA films. There is a balance between film thickness and swelling, as thicker films have poorer swelling capabilities however no delamination is observed after 16 hrs in the incubation medium, whilst thinner films have better swelling capabilities but delamination occurs over 16 hours in the incubation medium.

To chemically analyse hydrated hydrogel films comprised of a physically cross-linked pHEMA networks, either films of certain thicknesses ca. 50 nm or reduced incubation times of thinner films below 16 hrs may be needed.

CHAPTER 4 – DEVELOPING PREPARATORY PROTOCOL

4.1. AIM

We aim to develop a preparatory procedure for introducing hydrogels into a ToF-SIMS instrument for frozen hydrated 3D chemical analysis. In this study we compare the results of cryo-depth profiling a poly(2-hydroxyethyl methacrylate) hydrogel film prepared by 3 different methods. We show that the usage of a fast argon gas flow to physically remove frost formed on the sample surface subsequently produces good depth profiles, where the pHEMA film chemistry may be fully characterised in 3D using ToF-SIMS cryo-depth profiling in a frozen hydrated format. Using argon gas in an “air gun” style over the surface produces a sample where limited frosting of the sample surface is present and the film presents a steady state concentration of a polyatomic water cluster ($H_{13}O_6^+$) indicating that the film hydrated throughout.

4.2. INTRODUCTION

Due to ToF-SIMS being an ultra-high vacuum technique, analysis of hydrogels in a hydrated state presents a number of challenges in sample preparation and analysis. The analysis of cells/tissue and other hydrated materials including hydrogels has been a fervent research area in electron microscopy, where it is paramount that sample preparation does not induce significant physical changes. In the case of ToF-SIMS, physical and chemical changes must be minimised in sample preparation and analysis. Most frozen hydrated preparation techniques for cellular studies involve freeze drying the sample by snap freezing in a cryogenic liquid then ensuring vacuum sublimation of ice. With cryofixation the sample must be rapidly frozen so that vitrification of ice is promoted rather than crystalline ice formation, which may dehydrate or freeze fracture the sample. Recently, Schaepe *et al.* published a study comparing various cell preparation techniques mentioned above including frozen-hydrated¹⁴⁰, but prior to imaging they raised the temperature of the sample holder in order to sublimate water from the cell surfaces. This raises the question to what extent the sample can still be considered frozen-hydrated. Ice crystals could have formed as well given that the recrystallization temperature was exceeded. Furthermore, to our knowledge nearly all examples of freeze-fractured and frozen-hydrated imaging experiments in the literature are executed in 2D.

Literature on chemical characterisation of hydrogels is relatively sparse. Lukas *et al.* Published a study in 1996 on XPS surface analysis of two statistical copolymers¹⁹, poly(2-hydroxyethyl methacrylate-co-butyl methacrylate) (HEMA-co-BMA) and poly[2-(2-ethoxyethoxy)ethyl methacrylate-co-butyl methacrylate]

(EOEOEMA-co-BMA). This involved a “deep-freezing” technique where the samples were snap frozen in liquid nitrogen. This analysis was limited to the surface, whilst the preparation involved freeze drying in the entry chamber allowing ice sublimation to occur. Significant physical and chemical changes may have occurred in this manner, therefore the hydrogel may not be considered in a true “hydrated state”.

DJ Hook *et al.* also examined a dual XPS/ToF-SIMS study of hydrogel surfaces in a similar surface manner¹¹⁵. They investigated the surface changes upon hydration / dehydration of seven poly (allyl methacrylate-g-dimethylsiloxane), AMA-g-DMS, graft copolymers, in the first incidence of detection of high mass oligomeric ion distributions from hydrated (frozen) surfaces were found.

These reported studies are all limited to surface characterisation. Assessing the impact of hydrogel chemistry and biomolecules on cellular response ideally requires methods which allow characterisation without labels and can map species in 3D to follow biomolecules adsorbed to, and absorbed into, the porous hydrogel structure before and during culture. Here, We describe the development of a number of methods for introduction of pHEMA hydrogels into UHV for ToF-SIMS analysis.

4.3. RESULTS

To chemically analyse hydrogels by ToF-SIMS, hydrogels require a format that ensures dehydration associated physical changes in the sample under vacuum are minimised. Snap freezing cell and tissue samples has been well documented in literature in analysis of frozen cell samples by TEM/SEM. Hydrogels, similarly to cells and tissues, maintain a percentage of water which contributes to the overall composition of the gel.

Ideally snap freezing is performed *in situ* to prevent condensation of moisture on the sample surface. We investigated a number of approaches for entry. Four different methods of hydrogel sample preparation were performed in this study, where we found that the usage of a high flow of argon gas to evacuate the sample surface, prior to entry in the sample chamber and before evacuation of the ToF-SIMS analysis chamber was suitable to minimise frost formation on the sample surface and produce a full through thickness depth profile of a frozen hydrated pHEMA hydrogel.

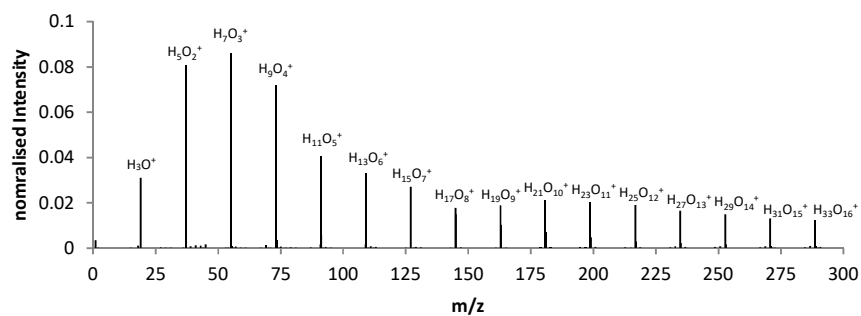
The results from the sample preparation method when the pHEMA film was cooled on the stage via a flow of liquid nitrogen are shown in **Figure 28a**. The surface spectra of the sample shows the presence of water by the projection of polyatomic water clusters ($H_{2n+1}O_n$) ranging from $n = 1-16$ indicative ice. The water clusters dominate the spectra with the absence of any organic peaks.

A secondary ion diagnostic of each component of the sample was selected to represent pHEMA ($C_6H_9O_2^+$ m/z 113.089), protonated water signifying ice ($H(H_2O)_6^+$ m/z 91.688) and the silicon substrate (Si^+ m/z 27.987). The graph was

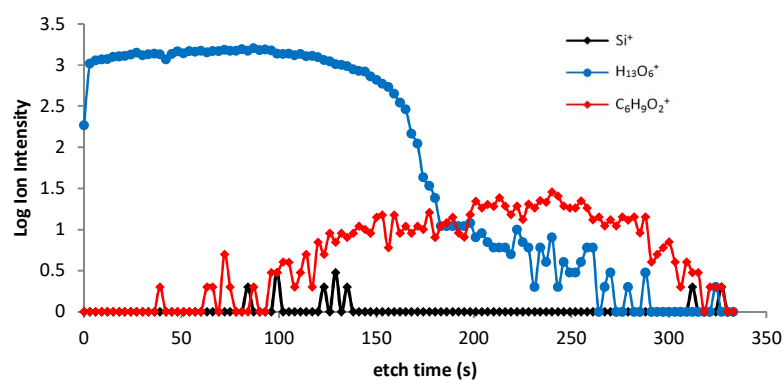
plotted on a logarithmic scale to better show the broad interface region between the ice and pHEMA. The through thickness profile is shown in *Figure 28b*. There appears to be a thick ice associated layer at the surface evidenced by the steady state ion intensity of $\text{H}_{13}\text{O}_6^+$ in the 1st 100 seconds of sputter etching. The signal intensity decays at the broad interface between ice and pHEMA. The silicon wafer secondary ion marker Si^+ is plotted as a background peak intensity as the interface between film and silicon wafer is not reached. The lateral distribution of $\text{H}_{13}\text{O}_6^+$ on the sample surface in *Figure 28b* revealed a differential distribution of ice of the surface, the total ion image also shows a rough topographic surface in *Figure 28c*.

COOLED IN CHAMBER

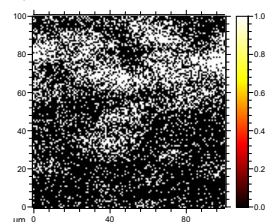
a)



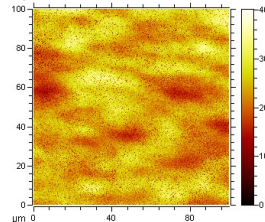
b)



c)



d)



e)



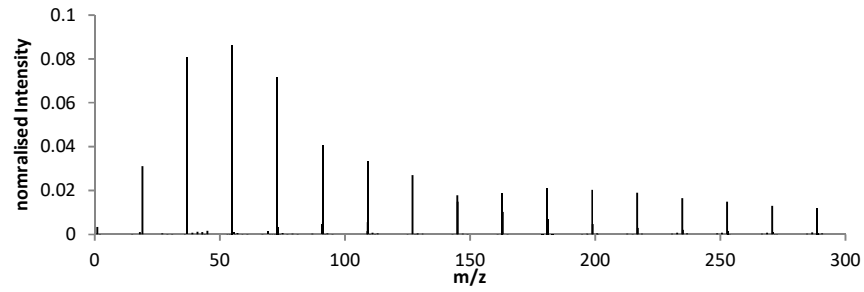
Figure 28 - Positive polarity ToF-SIMS spectra acquired from 5 scans of the pHEMA stage cooled hydrogel

A) a surface spectra, B) through thickness depth profile, C) surface images of $H_{13}O_6^+$ indicating ice distribution, d) total ion image of the surface, e) picture showing sample frosting on the surface

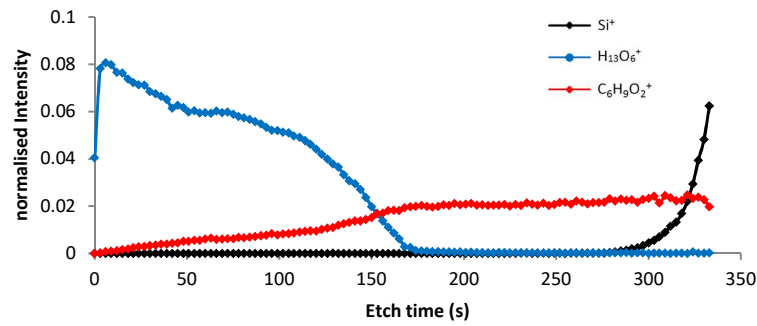
The results from the sample preparation method when the pHEMA hydrogel was snap frozen in liquid nitrogen prior to mounting on the precooled cryo-stage in the entry chamber are shown in **Figure 29**. Again, the surface spectra of the sample shows the presence of water by the projection of polyatomic water clusters ($H_{2n+1}O_n$) ranging from $n = 1-16$ indicative ice. The water clusters dominate the spectra with the absence of any organic peaks. The Ar-GCIB depth profile of the pHEMA film is shown in **Figure 29b**. The profile shows that a high ion intensity of $H_{13}O_6^+$ is present on the surface region, which degrades to background intensity after 150 seconds of etching. $C_6H_9O_2^+$ is present on the surface and increases to a steady state concentration after 180 seconds etching until the interface between pHEMA and silicon wafer is reached.

SAMPLE PLUNGE FROZEN THEN STAGE MOUNTED

a)



b)



c)

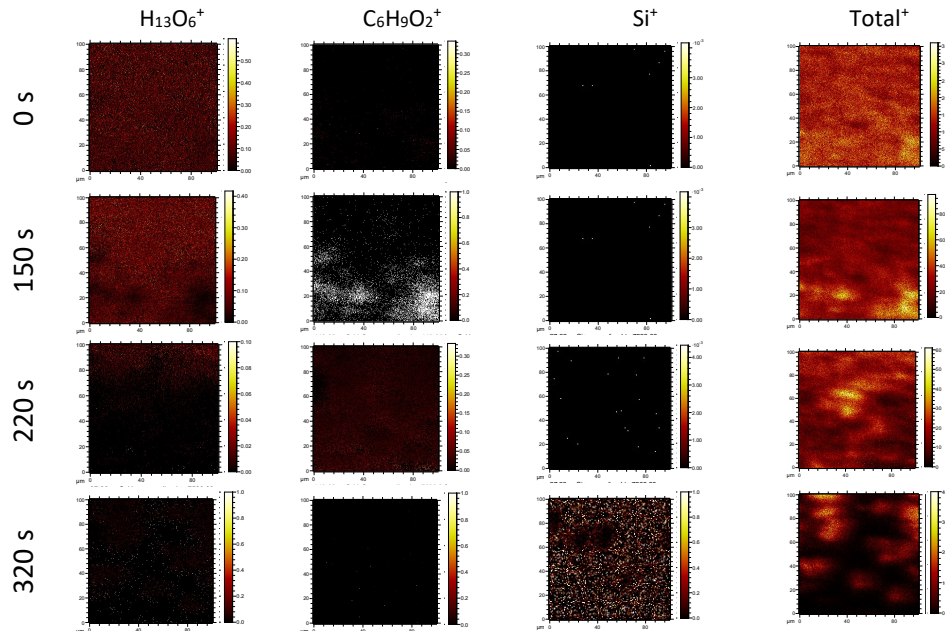


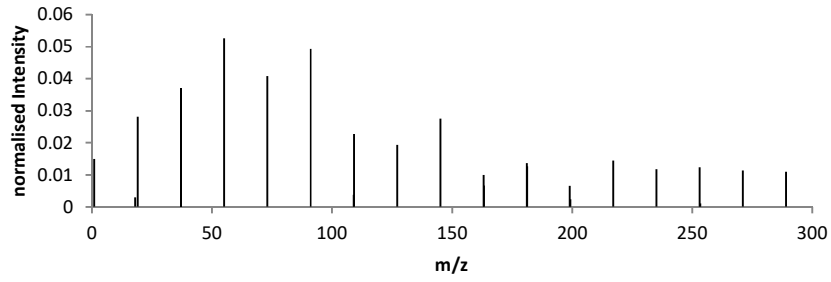
Figure 29 - positive polarity TOF-SIMS spectra acquired from 5 scans of the phema snap frozen hydrogel surface

a) A surface spectra, b) through thickness depth profile, c) surface images of $H_{13}O_6^+$ indicating ice distribution $C_6H_9O_2^+$ indicating phema distribution, Si^+ indicating silicon wafer distribution and total ion image of the surface.

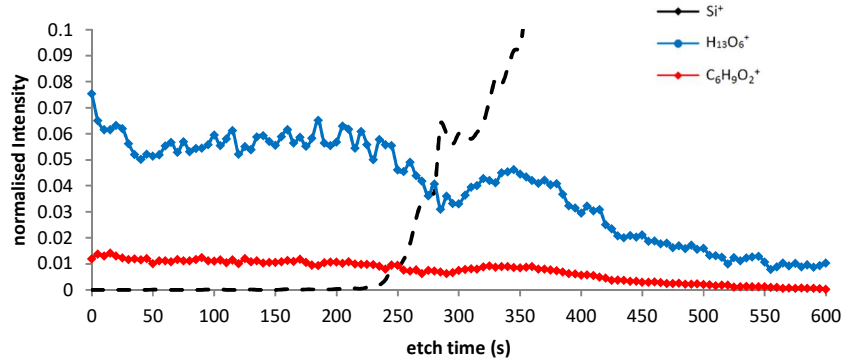
Analysis of the surface of the stage snap frozen pHEMA film is shown in **Figure 30a**. The surface spectra of the sample shows the presence of water by the projection of polyatomic water clusters ($H_{2n+1}O_n$) ranging from $n = 1-16$ indicative ice. $C_6H_9O_2^+$ and other polymer associated fragments are also present on the surface. The through thickness profile is shown in **Figure 30b**. Ice is shown in blue, pHEMA in red and silicon as a dashed black line. The normalised Intensity of $H_{13}O_6^+$ is elevated at the surface and decreases to a steady state at 0.06 normalised ion intensity through the profile to the interface between gel and silicon wafer at 220 second. The interface between gel and silicon wafer is broad, indicating that there is not a defined layer. The lateral distribution of ice and the interface between gel and silicon wafer shown in **Figure 30c** showing a uneven distribution in this scan.

SAMPLE STAGE FROZEN

a)



b)



c)

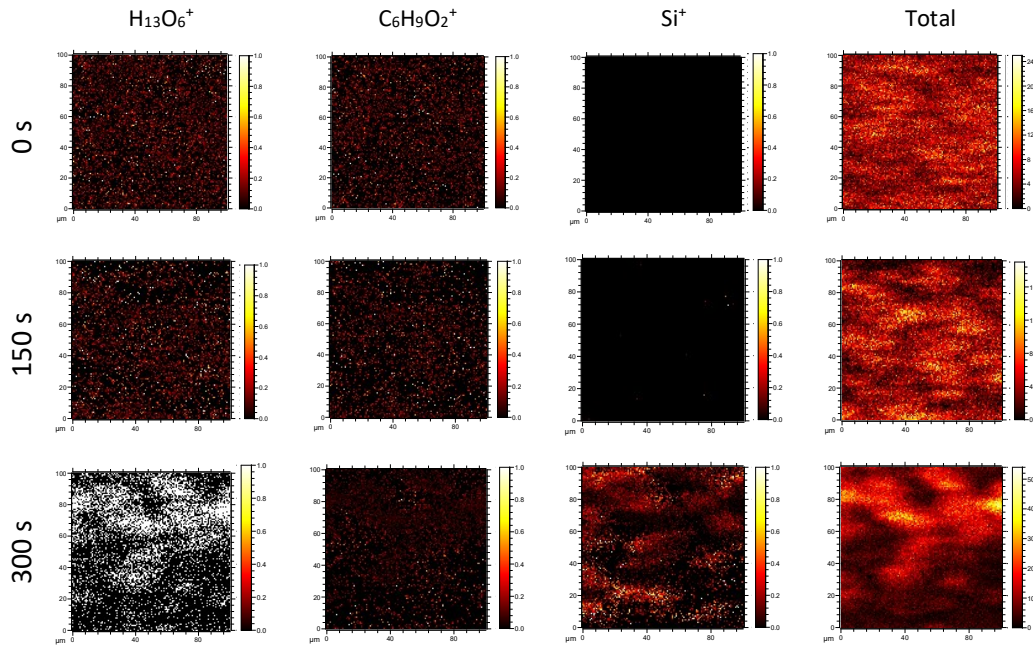


Figure 30 - positive polarity ToF-SIMS spectra acquired from 5 scans of the pHEMA snap frozen hydrogel surface

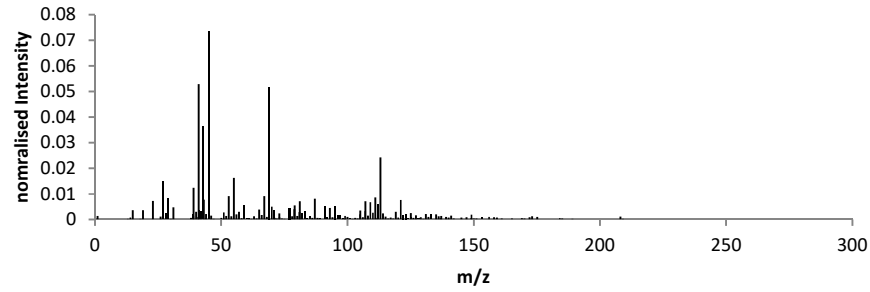
a) a surface spectra, b) through thickness depth profile, c) surface images of $H_{13}O_6^+$ indicating ice distribution $C_6H_9O_2^+$ indicating pHEMA distribution, Si^+ indicating silicon wafer distribution

To minimise the formation of surface ice (frost) we explored the use of argon in an air gun style to evacuate the sample surface post snap freezing. Initial analysis of the surface shown in **Figure 32a** identified the presence of organic peaks, as well as the isolation of polyatomic water clusters ranging from $n = 4-16$, indicating the presence of ice as well as pHEMA at m/z 113.16. Mass peaks attributed to $n = 1-3$ were also observed however, the peak shape for the lower range protonated water clusters showed peak contribution from organic species, therefore could not be used independently for qualification of ice.

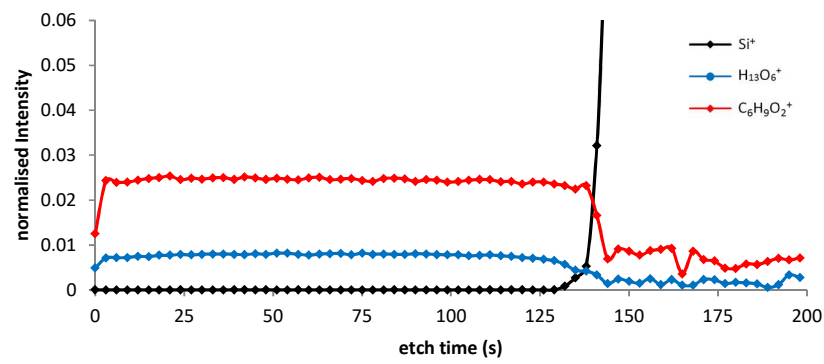
The through thickness depth profile of the pHEMA hydrogel shown in **Figure 32b** displays a steady state ion intensity with respect to pHEMA ($C_6H_9O_2^+$) and water/ice ($H_{13}O_6^+$) from 0 to 120 seconds etching, where up the interface between silicon and the hydrated pHEMA film is reached where the signals degrade. The interface region is narrow (5 seconds etching) indicating a good depth resolution between the two layers. The lateral distribution of $H_{13}O_6^+$ in the surface region in **Figure 32c** shows a uniform distribution with an absence of features, the same is observed in the case of pHEMA ($C_6H_9O_2^+$).

STAGE FROZEN ARGON-GUN

a)



b)



c)

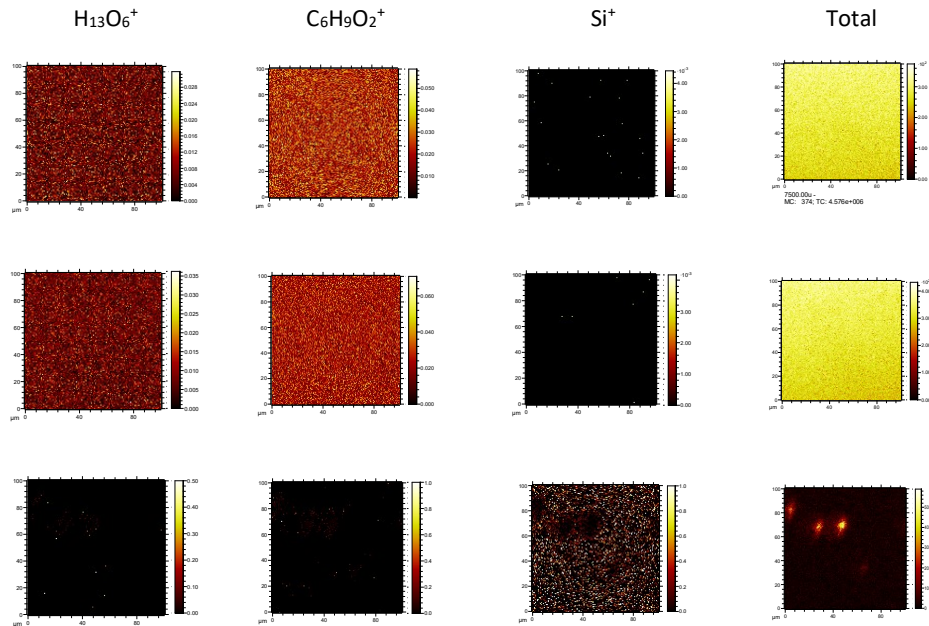


Figure 31 - positive polarity ToF-SIMS spectra acquired from 5 scans of the phema snap frozen hydrogel surface

a) a surface spectra, b) through thickness depth profile, c) surface images of $H_{13}O_6^+$ indicating ice distribution $C_6H_9O_2^+$ indicating phema distribution, Si^+ indicating silicon wafer distribution

Table 8 - Positive secondary ions reported from depth profiling pHEMA hydrogel.

	m/z	Structure	Deviation (ppm)
	19.020	$\text{H}(\text{H}_2\text{O})^+$	39.3
	22.999	Na^+	26.5
	27.034	C_2H_3^+	14.3
Silicon Wafer	27.987	Si^+	-22.1
	29.047	C_2H_5^+	3.3
	39.030	C_3H_3^+	3.5
	41.041	C_3H_5^+	26.1
	43.022	$\text{C}_2\text{H}_3\text{O}^+$	30.4
	45.040	$\text{C}_2\text{H}_5\text{O}^+$	42.1
	53.043	C_4H_5^+	22.8
	55.069	C_4H_7^+	31.1
	69.044	$\text{C}_4\text{H}_5\text{O}^+$	43.1
	87.051	$\text{C}_4\text{H}_7\text{O}_2^+$	35.8
	91.688	$\text{H}(\text{H}_2\text{O})_5^+$	-47.1
Water (n=6)	109.086	$\text{H}(\text{H}_2\text{O})_6^+$	8.3
pHEMA	113.089	$\text{C}_6\text{H}_9\text{O}_2^+$	51.1
	127.096	$\text{H}(\text{H}_2\text{O})_7^+$	4.9
	145.113	$\text{H}(\text{H}_2\text{O})_8^+$	62.9
	182.123	$\text{H}(\text{H}_2\text{O})_{10}^+$	-31.7
	199.130	$\text{H}(\text{H}_2\text{O})_{11}^+$	2.4

4.4. DISCUSSION

We first explored cryo-cooling the pHEMA hydrogel whilst mounted on the ToF-SIMS cryo-stage, by snap freezing the sample through pouring a steady stream of liquid nitrogen onto the sample mounted on the stage. This was performed as sample mounting once frozen is a time consuming process, and the length of time the frozen sample is present in ambient atmospheric conditions would comprise the sample viability allowing frost to condense on the sample. The entry and analysis chamber were pre-cooled to a cryogenic temperature of -120°C prior to entry and evacuation as this maintains the sample temperature below the point where the rate of sublimation of ice under vacuum increases significantly (-80 to -110°C), as reported in literature⁸⁴.

The absence of organic fragments on the surface and the isolation of purely polyatomic water clusters indicated that an ice layer was present on the surface of the pHEMA film suggesting that the thickness of the ice layer on the surface is above the average analysis depth of 2 nm given for ToF-SIMS. Upon inspection of the sample in the analysis chamber there was visible frosting on the sample surface which suggested that the observed topography in the lateral ion images of $\text{H}_{13}\text{O}_6^{+}$ was associated with frosting of the sample as a result of the exposure of the sample above the allotted temperature of -120°C or condensation of water in the atmosphere on the sample. The broad interfacial region between the pHEMA film and ice indicated that sputter induced roughening was occurring. The silicon wafer is was not accessed in the profile, so the decrease in signal intensities of $\text{H}_{13}\text{O}_6^{+}$ and $\text{C}_6\text{H}_9\text{O}_2^{+}$ was most likely attributed to roughening on the surface as a factor of frost formation developing differences in feature height in the sample

through roughening from Argon etching. The poor depth profile for the mixed material is which indicated that this preparatory procedure was not suitable for introducing frozen hydrated samples. This failure may be attributed to the differences in temperature of the cryogen and the pHEMA hydrogel, causing sample insulation or Leidenfrost effect. If a liquid is in near contact with a mass significantly hotter than the liquid's boiling point, it produces an insulating vapour layer keeping that liquid from boiling rapidly. Due to this 'repulsive force', a droplet hovers over the surface rather than making physical contact with it. This results in slow cooling and may contribute to condensation of atmospheric water on the sample surface.

As frosting was observed on the sample which may have been a factor of the method of freezing, plunge freezing the sample then mounting was performed as the sample insulation is far poorer if the sample is immersed in the cryogen, i.e. snap frozen, rather than frozen by a stream of liquid nitrogen. The silicon wafer with dip coated pHEMA film post incubation was plunged in liquid nitrogen for 5 minutes until the sample temperature was of that of the liquid nitrogen, evidenced by the absence of liquid nitrogen boiling. Initial inspection of the sample surface prior to plunge freezing revealed a frost free surface. However from sample transfer to the precooled entry chamber, frosting occurred, this was further exacerbated by the length of time taken to mount the sample, as well as the low temperature environment in the entry chamber being exposed to the ambient atmosphere upon opening the entry chamber. This may have contributed or caused condensation of water on the sample surface then freezing,

generating frost. The depth profile of this respective entry method suggest that there is a differential distribution of ice on the sample surface, indicated by the measurement of secondary ions attributed to organic pHEMA, as well as ice. If the ice film at the surface was present as a vitrified layer, rather than frosted. A uniform steady state concentration would be present on the Initial surface region, which would degrade into the bulk gel as the proportion of organic material in the film increases; however, this was not observed. The 2D ion images of $\text{C}_6\text{H}_9\text{O}_2^+$, $\text{H}_{13}\text{O}_6^+$ and Si^+ at the interface regions certainly suggest the distribution of ice is uneven on the surface.

We concluded that the time the cryo-snap frozen sample/cooled cryostages was exposed to ambient conditions was the catalyst for frost formation on the sample. To reduce this time taken we explored plunge freezing the cryo-stage whilst the sample was mounted. This would allow us to transfer the sample rapidly to the precooled entry chamber whilst minimising the time the precooled entry chamber was exposed to the ambient atmosphere. The sample transfer from cryogen to entry chamber and evacuation on the entry chamber was as rapid as possible, c.a. 8 seconds, which produced a surface where minimal frosting was visible. Observation of the sample within the entry chamber indicated that the surface was visibly flat and minimal parts of the sample showed crystallisation of ice on the surface/frost. The depth profile indicated that the film was hydrated, with steady state concentration of both pHEMA and ice attributed ions, however the interface width is broad, suggesting no defined separation between the pHEMA/silicon wafer substrate. The elevated ion intensity with respect to $\text{H}_{13}\text{O}_6^+$

suggests a surface enrichment of ice, the lateral distribution at the interface suggest the distribution of this surface enriched layer is not uniform, which may be contributing to the broad interface.

The use of a gas to remove aggregate frost deposits from cooling-coil batteries in freezing plants was patented in 1986 (US 4570447 A)¹⁴¹. In detail a current of compressed air is directed towards and brought to sweep over the cooling-coil batteries. Here, frost build up was removed with a high flow rate of argon to clear the surface of ice crystals which accumulated during the transfer between plunging with liquid nitrogen to the pre-cooled sample entry chamber.

A time of 4 seconds argon flow over the sample surface was found to be sufficient to remove surface ice without inducing visible changes in the sample surface. The total sample transfer from cryogen to the precooled entry chamber was assessed as 8 seconds. Purging of the entry chamber was also employed with nitrogen to eliminate the condensation of species on the sample surface. Maintaining a frozen sample state between the liquid nitrogen plunge and the analysis chamber via the pre-cooled sample entry chamber was a significant consideration as the sublimation rate of ice in the vacuum chamber increases as sample temperature rises. Sample mounting onto the IonTOF cryo stage was a time consuming, thus plunge freezing the whole stage assembly allowed immediate transfer of samples to the precooled entry chamber as this did not have any measureable detrimental effect on the instrument vacuum. Previous works by Gemmei-Ide *et al* among others report a target temperature range of -70°C to -110°C for sample preparation of frozen samples for vacuum

analysis, and state that if the sample temperature exceeds -70°C ice crystallisation can occur ^{142,143}. Furthermore, the sample transfer time from liquid nitrogen immersion to the entry chamber was also performed rapidly as otherwise the accumulation of frost on the sample surface occurs when the sample is exposed to the ambient atmosphere.

The isolation of organic species with polyatomic water clusters on the surface as well as the bulk pHEMA suggested a hydrated state on the surface as well as in the film, and that the thickness of surface ice was below 2nm. The marker for pHEMA ($\text{C}_6\text{H}_9\text{O}_2^+$) did not decay in its intensity from the surface to the bulk, similarly $\text{H}_{13}\text{O}_6^+$. The profiles ion intensity with respect to $\text{H}_{13}\text{O}_6^+/\text{C}_6\text{H}_9\text{O}_2^+$ are stable and surprising good. This indicated to us that the preparatory procedure was suitable for introduction of frozen-hydrated hydrogels for 3D ToF-SIMS depth chemical analysis.

4.5. CONCLUSION

A sample preparation procedure using argon gas in an “air gun” style to remove the surface frost accumulated on the cooled sample surface on exposure to ambient atmosphere yielded interesting results. It has been demonstrated that poly(2-hydroxyethyl methacrylate) hydrogels can be successfully chemically characterised as frozen hydrated films by utilising 10 KeV Ar_{1500}^+ cluster sputtering by ToF-SIMS. The methodology allows full through-thickness chemical characterisation of the pHEMA hydrogel by ToF-SIMS.

This method of 3D chemical characterisation of frozen hydrated samples is of particular importance as it provides a way of effective full through-thickness chemical characterisation of hydrogels which is of importance to the biomedical research field.

CHAPTER 5 – ToF-SIMS/XPS DEPTH PROFILING OF FROZEN-HYDRATED HYDROGELS

5.1. AIM

We aim to show that the cryogenic sample entry procedure developed in chapter 4 is appropriate for introducing poly(2-hydroxyethyl methacrylate)/Lysozyme hydrogels into a ToF-SIMS instrument for frozen hydrated 3D chemical analysis. Furthermore, show that that lysozyme location may be mapped in hydrogel films in a frozen-hydrated format. We also aim to show that the sample entry procedure is appropriate for introducing hydrogels into a XPS instrument for frozen hydrated 3D chemical analysis, as developed for the ToF-SIMS.

We compare the results of ToF-SIMS/XPS cryo-depth profiling of the same sample; a sample of poly(2-hydroxyethyl methacrylate)/Lysozyme hydrogel, showing that the method of preparing pHEMA hydrogel films and introducing them into the ultra-high vacuum (UHV) instrument provides good quality ToF-SIMS/XPS depth profiles.

Lastly we compare ToF-SIMS/XPS data sets of the lysozyme/pHEMA hydrogel, and show a good correlation between the normalised intensities of lysozyme associated markers with the values relating to protein distribution acquired from our XPS analyses.

5.2. INTRODUCTION

Hydrogels have been used extensively in bioengineering as artificial cell culture supports. Investigation of the interrelationship between cellular response to the hydrogel and its chemistry, ideally requires methods which allow characterisation without labels and can map species in 3D to follow biomolecules adsorbed to, and absorbed into, the open structure before and during culture.

Time-of-flight secondary ion mass spectrometry has the potential to be utilised for through thickness characterisation of hydrogels. Hydrogel characterisation in 3D is currently unexplored. The effect of temperature on depth profiling polymers has been probed by Piwowar *et al*²². observing increased ion yields at 100K as compared to 293.15K in the high mass region for amino acid and polypeptide secondary ions. However, all cryogenic analysis formats for ToF-SIMS analysis of hydrogels to date either involve freeze drying hydrogel films in vacuum¹⁰⁵ or are limited to analysis of the surface (2 nm)^{115,144}.

We apply the simple sample preparation procedure in chapter 4 and subsequent low temperature analysis on a poly(2-hydroxyethyl methacrylate) (pHEMA) film where a model protein (lysozyme) is incorporated using 2 methods to demonstrate how protein distribution can be visualised in a label free manner.

Biomolecule mobility within hydrogels is strongly dependent on size, isoelectric point and charge, solubility and method of introduction, i.e. whether the biomolecule is included in the gelation or introduced post gelation⁶. We hypothesise that different protein incorporation methods lead to different protein

distribution throughout the film and that we can measure this by creating depth profiles of protein concentrations.

A comparison of lysozyme incorporation is made between the situation where the protein is present in a polymer dip coating solution and where lysozyme is in an aqueous medium in which the film is incubated. The lysozyme distribution through the pHEMA hydrogel films can be determined using the intensity of a characteristic amino acid secondary ion fragment $C_4H_6N_2^+$.

While ToF-SIMS provides the ability to elucidate the chemical composition of a sample, it does not currently allow for quantitative measurement from ToF-SIMS data acquisition alone. The measured ion intensity may be strongly influenced by the electronic state of the material, or matrix effect. The matrix may influence SIMS measurement in two ways: the sputter yield of secondary ions which is the least dependent on the matrix effect, and Ionisation probability which can vary significantly for different sample matrices. This effect may be minimised through analysis of sputtered neutral ions rather than secondary ions, via post-ionisation secondary neutral mass spectrometry¹²⁰.

ToF-SIMS can be quantitative if standards of known chemical amounts and distribution are calibrated to the ion intensities of secondary ions specific to chemical moiety. To quantitatively chemically analyse materials such as polymer films through ToF-SIMS usually employs XPS in tandem to gain molecular and relative elemental amounts from the analysed material¹²⁹. XPS is able to provide quantitative information on the relative amount of the studied element providing

the elements or chemical functionalities of interest are distinct from the material and present in sufficient amounts to be detected next to the surrounding material.

To this end XPS may be useful for characterising hydrogel surface and bulk chemistry as similarly to ToF-SIMS analysis takes place in a UHV with the ability to perform low temperature analysis. The majority of papers reporting XPS studies of hydrogels employ ambient analysis conditions, where the low vacuum pressure causes water desorption and dehydration of the gel changing the gel composition^{34,145,146}. There have been examples of cryogenic analysis of hydrogels through XPS, however these sample formats allow the prepared hydrogel to freeze dry in the low vacuum entry chamber prior to analysis¹⁹. All studies so far have been limited to the analysis of the surface, to date, no depth profiling by XPS has been performed on hydrogels.

We apply the procedure developed in chapter 4 for XPS analysis of hydrogels, demonstrating this on a poly(2-hydroxyethyl methacrylate) (pHEMA) film where a model protein (lysozyme) is incorporated to demonstrate how protein distribution can be determined. The lysozyme distribution through the pHEMA hydrogel films can be determined using the atomic concentration of nitrogen. Two frozen hydrated samples are compared in this study, where a pHEMA film immersed in lysozyme solution is compared to a pHEMA film immersed in water.

Lastly, we compare the two XPS/ToF-SIMS data sets of the same pHEMA/Lysozyme samples showing a close correlation between normalised ToF-SIMS ion intensities ($\text{C}_4\text{H}_6\text{N}_2^+$) and Nitrogen/carbon ratios, demonstrating the mapping of lysozyme in frozen-hydrated pHEMA hydrogels may be quantitatively determined.

5.3. RESULTS

Three frozen hydrated samples are compared in this first section with the developed argon air gun preparatory procedure, including a pHEMA film, a pHEMA film immersed in lysozyme solution and lysozyme mixed with the pHEMA dip solution used to form a film. The positive polarity secondary ion spectra acquired using Bi_3^{++} from the surface of the samples are presented in **Figure 32**. All the spectra are dominated by secondary ion peaks characteristic of pHEMA, such as at $m/z = 39, 41, 43, 45$ and 69 (labelled with red markers in **Figure 32**) the corresponding formulas for which are shown in **Table 9**. Protonated water clusters at $m/z = 91, 109, 127, 145$ and 199 representative of $\text{H}(\text{H}_2\text{O})_5^+$, $\text{H}(\text{H}_2\text{O})_6^+$, $\text{H}(\text{H}_2\text{O})_7^+$, $\text{H}(\text{H}_2\text{O})_8^+$ and $\text{H}(\text{H}_2\text{O})_{11}^+$ respectively were additionally observed (highlighted with blue markers in **Figure 32**). The positive histidine secondary ion is used as a marker for lysozyme by the $\text{C}_4\text{H}_6\text{N}_2^+$ peak at $m/z = 82$ (green marker in **Figure 32**) as this has been reported as a positive marker for lysozyme in literature¹⁴⁷. A peak close to $\text{C}_4\text{H}_6\text{N}_2^+$ region was detected at of $m/z = 82.182$ was detected in the pure pHEMA sample, however this was attributed to pHEMA ($\text{C}_4\text{H}_6\text{O}_2^+$) rather than sample contamination as no CH_4N^+ or NH_4^+ ions were detected. The mass peak at $m/z = 82.047$ was also absent from this control sample.

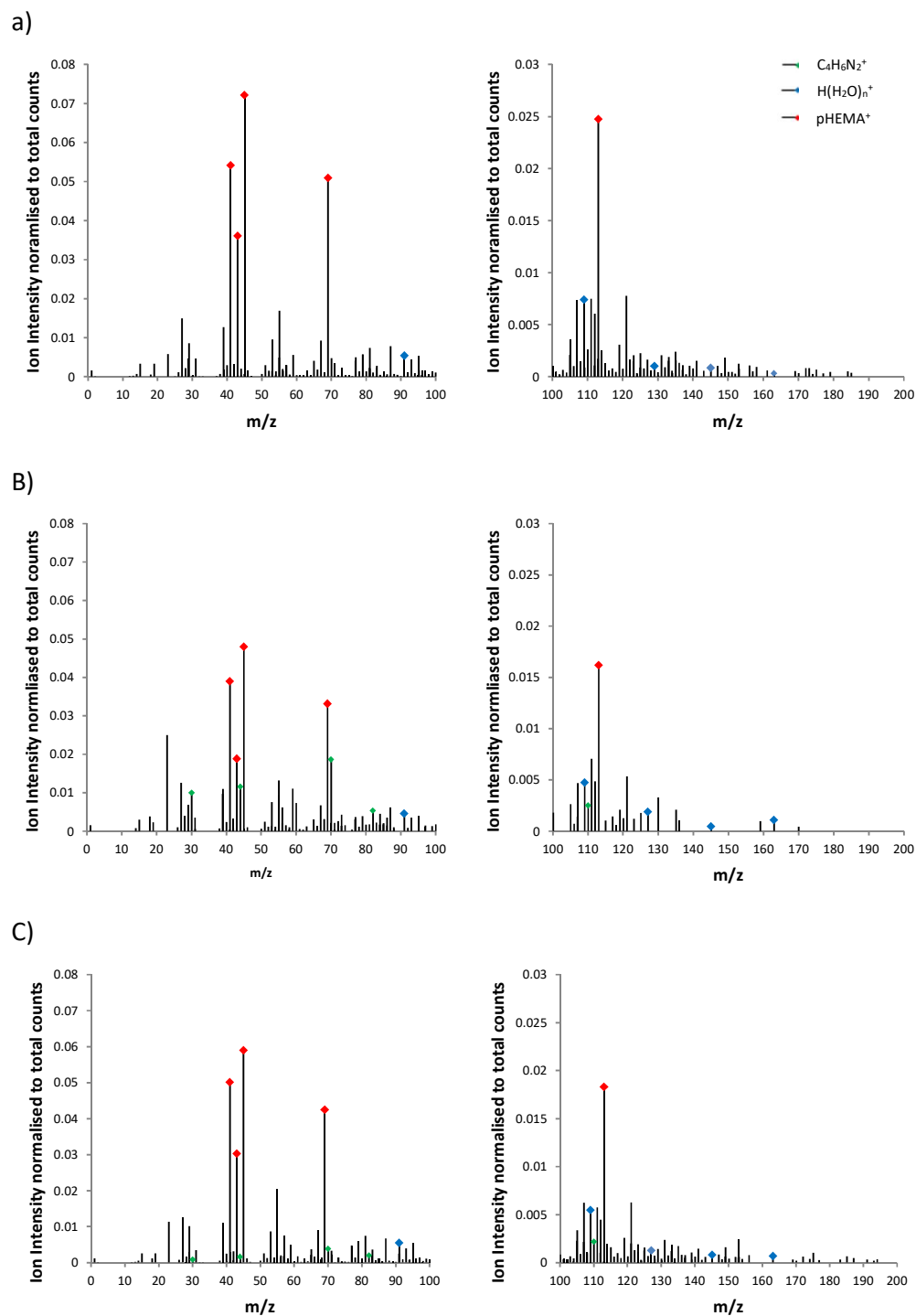


Figure 32 - Positive polarity ToF-SIMS spectra combined from 5 scans of the hydrogel surface with and without lysozyme.

A) a pHEMA film, B) a pHEMA hydrogel incubated in 1% wt/v lysozyme in water for 16 hours at 20°C and C) a silicon wafer dip coated in an ethanoic solution of 6% wt/v pHEMA/1% wt/v lysozyme. Peaks attributed to pHEMA are displayed in red, lysozyme in green and water in blue.

A representative diagnostic secondary ion of each component of the sample was selected for pHEMA ($\text{C}_6\text{H}_9\text{O}_2^+$), lysozyme ($\text{C}_4\text{H}_6\text{N}_2^+$), protonated water signifying ice ($\text{H}(\text{H}_2\text{O})_6^+$) and the silicon substrate (Si^+). Depth profiles were collected for the pHEMA film, a pHEMA film incubated in lysozyme solution and a pHEMA/lysozyme film produced by dip coating into a combined lysozyme/pHEMA coating solution shown in **Figure 33 a, b and c** respectively. The depth profile of the pHEMA film with water (**Figure 33a**) suggests a surface enrichment of water and depletion of pHEMA evident in the ion intensity shifts from the first 10 seconds of the profile. The intensity of the fragment representing these components then remains constant until the relatively sharp pHEMA-silicon interface observed after 120 seconds of sputtering.

Beyond this point the spectrum is dominated by the Si^+ peak of the silicon wafer substrate at much lower overall intensity compared to the polymer film. As can be seen in **Figure 33**, the depth profile for the hydrogel film incubated in a lysozyme solution, both the $\text{C}_6\text{H}_9\text{O}_2^+$ and $\text{H}(\text{H}_2\text{O})_6^+$ ion intensities significantly increase from the surface compared to the bulk of the film. The $\text{C}_4\text{H}_6\text{N}_2^+$ ion, however, displays relatively a steady state ion intensity throughout the profile until reaching the interface between silicon wafer and hydrogel after 110 seconds of sputtering. A gradual decrease in the $\text{C}_6\text{H}_8\text{O}_2^+$ ion intensity is observed, with no steady state being achieved. Decreasing ion intensity was observed for 5 repeat samples, demonstrating that this effect is reproducible. The depth profile for the hydrogel film produced from dip coating a silicon wafer in a pHEMA/Lysozyme dip solution

displays a more pronounced increase in the lysozyme secondary ion marker ($\text{C}_4\text{H}_6\text{N}_2^+$) at the surface (*Figure 33b*).

This surface enrichment effect was observed over 5 samples, suggesting repeatability. The relative intensities of the $\text{C}_6\text{H}_8\text{O}_2^+$ and $\text{H}(\text{H}_2\text{O})_6^+$ ions increase from the surface, reaching a steady state intensity after 60 seconds of sputtering. The relative ion intensity of the $\text{C}_4\text{H}_6\text{N}_2^+$ ion (400) at the surface is reduced to a steady state intensity, 200) in the bulk of the film.

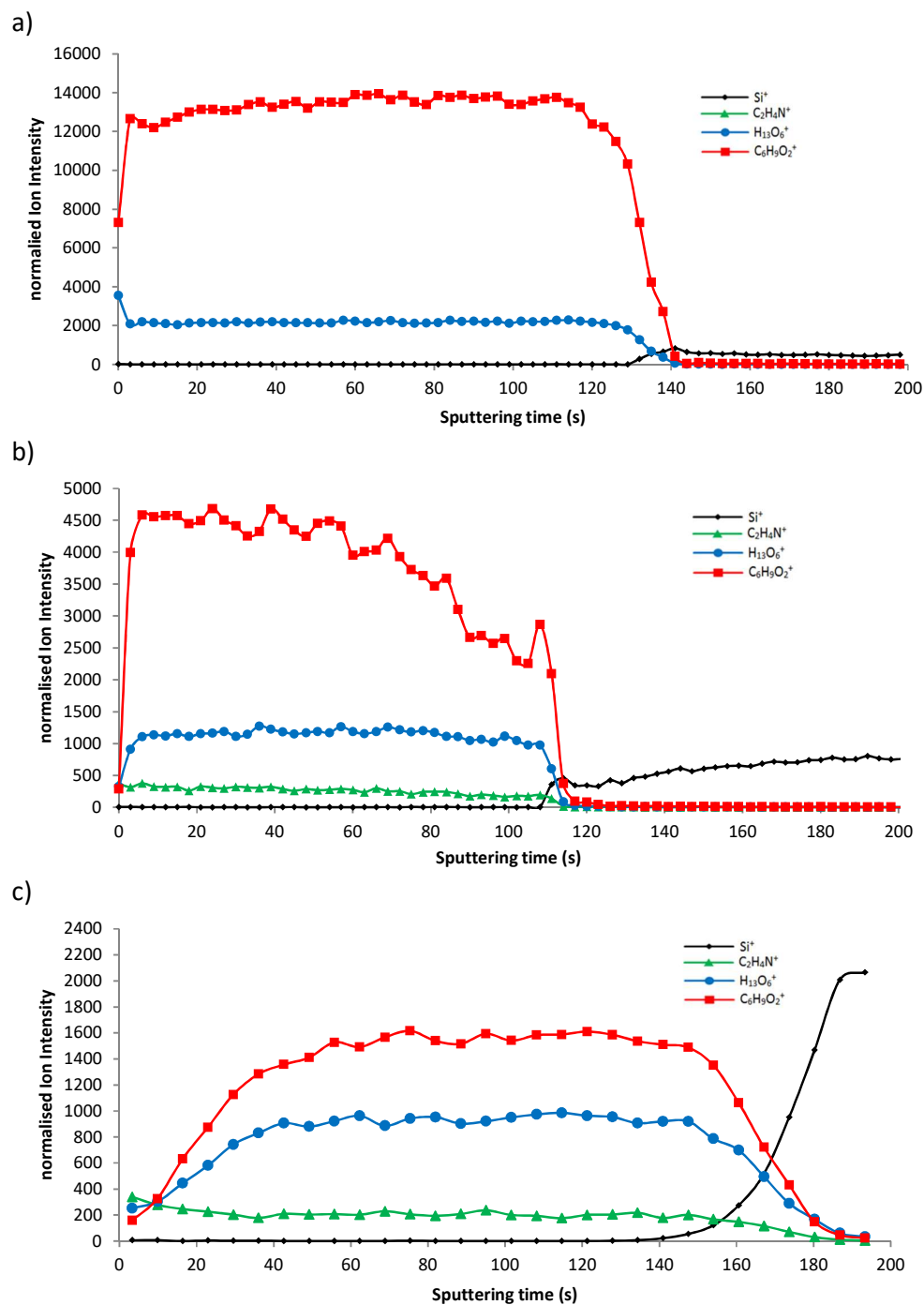
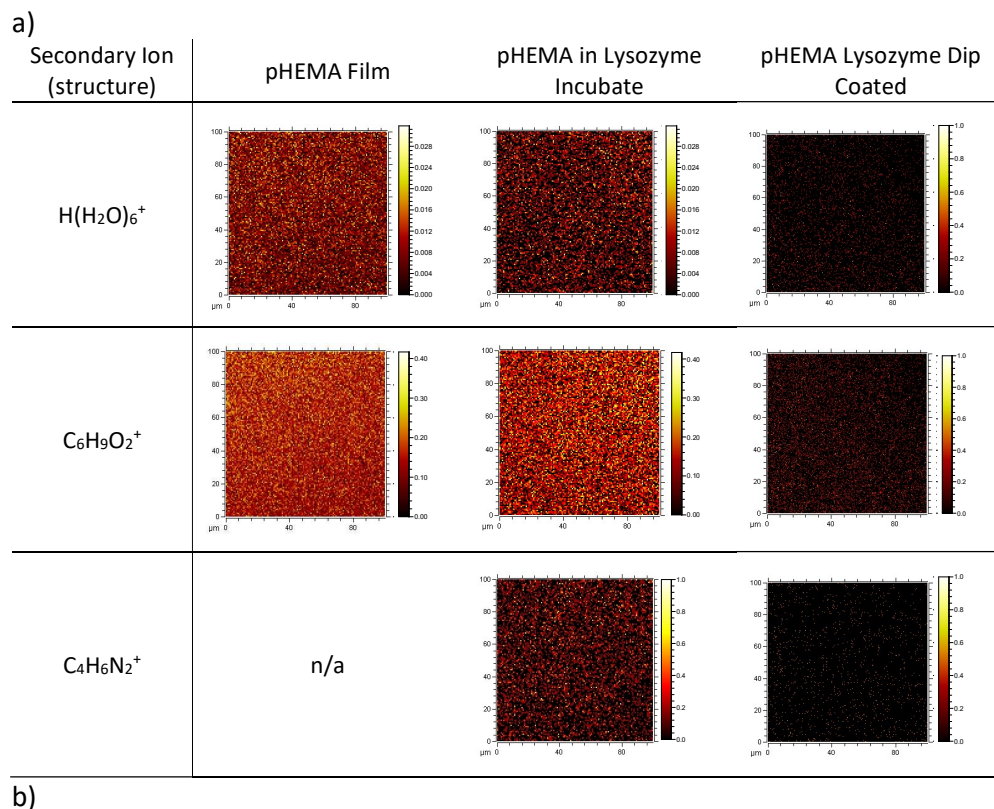


Figure 33 - ToF-SIMS depth profiles of pHEMA hydrogels incubated in water of water/lysozyme for 16 hours at 20°C.

A) a pHEMA film, B) a pHEMA hydrogel incubating the pHEMA films in 1% wt/v lysozyme in water for 16 hours at 20°C and C) a silicon wafer dip coated with a 6% wt/v pHEMA/1% wt/v lysozyme. Peaks attributed to pHEMA are displayed in red, lysozyme in green, ice in blue and silicon by the black dashed line.

The lateral distribution of the selected secondary ion fragments (*Figure 34a*) are uniformly distributed on the surface. The 3D render in *Figure 34b* for the pHEMA/Lysozyme dip coated solution followed by incubation in water displays that lysozyme is enriched the gel surface by a higher degree of green markers ($C_4H_6N_2^+$).



b)

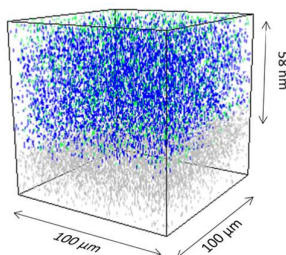


Figure 34 - Lateral distribution of secondary ion of the three different pHEMA sample formats with and without lysozyme, and distribution of lysozyme in 3D

a) Positive secondary ion maps from depth profiles of pHEMA hydrogels showing lateral distribution (X/Y coordinates) acquired at 60 seconds of sputtering. b) 3D render of pHEMA/lysozyme film prepared by dip coating in lysozyme/pHEMA solution. $C_4H_6N_2^+$ (Lysozyme) is represented in green, $H(H_2O)_6^+$ (ice) in blue and Si^+ (silicon wafer) in grey. The hydrogel z thickness represents the gel in a dry state, therefore will be an underestimate of the true thickness.

Table 9 - Positive secondary ions reported from depth profiling pHEMA hydrogels

Mass resolution and deviation is calculated by the division of peak intensity by peak height

	m/z	Structure	Assignment Deviation (ppm)		
			pHEMA	(pHEMA/lysozyme)	pHEMA/Lysozyme
	18.038	NH ₄ ⁺	28.2	32.2	40.1
	19.020	H(H ₂ O) ⁺	39.8	35.2	33.5
	22.999	Na ⁺	27.0	83.7	61.1
	27.034	C ₂ H ₃ ⁺	14.8	10.8	13.9
Silicon Wafer	27.987	Si ⁺	-22.9	-36.4	-
	29.047	C ₂ H ₅ ⁺	2.7	32.2	77.3
	30.023	CH ₄ N	-	12.4	11.9
	39.030	C ₃ H ₃ ⁺	5.7	5.4	20.5
	41.041	C ₃ H ₅ ⁺	26.5	4.3	50.0
	43.022	C ₂ H ₃ O ⁺	28.5	27.6	52.3
	44.218	C ₂ H ₆ N ⁺	-	-2.2	-4.6
	45.040	C ₂ H ₅ O ⁺	39.3	33.0	67.8
	53.043	C ₄ H ₅ ⁺	22.2	18.5	14.4
	55.069	C ₄ H ₇ ⁺	34.3	33.3	35.6
	69.044	C ₄ H ₅ O ⁺	43.4	32.2	68.9
	70.712	C ₄ H ₈ N ⁺	-	10.7	21.1
Protein (His)	82.047	C ₄ H ₆ N ₂ ⁺	-	-61.5	-48.5
	87.051	C ₄ H ₇ O ₂ ⁺	37.4	31.2	59.9
	91.688	H(H ₂ O) ₅ ⁺	-48.2	-56.3	-34.6
Water (n=6)	109.086	H(H ₂ O) ₆ ⁺	8.7	-2.0	24.0
	110.08	C ₅ H ₈ N ₃ ⁺	-	17.4	31.2
pHEMA	113.089	C ₆ H ₉ O ₂ ⁺	51.2	39.4	73.3
	127.096	H(H ₂ O) ₇ ⁺	4.3	0.2	4.4
	145.113	H(H ₂ O) ₈ ⁺	64.5	52.0	65.8
	182.123	H(H ₂ O) ₁₀ ⁺	-29.3	-36.6	-37.4
	199.130	H(H ₂ O) ₁₁ ⁺	0.9	6.3	-7.8

Table 10 - ion intensity ratios at steady state intensity in depth profile, $n=5$. standard deviations presented in parentheses.

	$\text{H}(\text{H}_2\text{O})_6^+ / \text{C}_6\text{H}_9\text{O}_2^+$	$\text{C}_4\text{H}_6\text{N}_2^+ / \text{H}(\text{H}_2\text{O})_6^+$	$\text{C}_4\text{H}_6\text{N}_2^+ / \text{C}_6\text{H}_9\text{O}_2^+$
1) pHEMA	0.18 (0.013)	-	-
2) lysozyme incubated pHEMA	0.29 (0.025)	0.32 (0.038)	0.094 (0.038)
3) pHEMA mixed with lysozyme before film formation	0.47 (0.076)	0.39 (0.089)	0.19 (0.068)

We had established that meaningful information could be derived from ToF-SIMS depth profiling frozen-hydrated hydrogels. We then attempted to sputter depth profile the same sample type through cryo-XPS in a frozen-hydrated format. A sample of poly(lactic-co-glycolic)acid was first sputter depth profiled in a non-cryogenic state using argon gas clusters as a pilot study to assess the capability of generating reliable data in depth profiling polymer based materials. The chemical structure of PLGA is shown in **Figure 35** including carbon and oxygen environments.

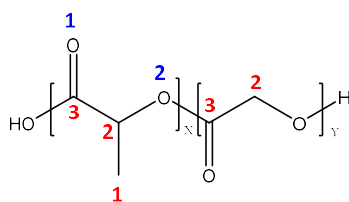


Figure 35 - Chemical structure of PLGA indicating carbon and oxygen environments.

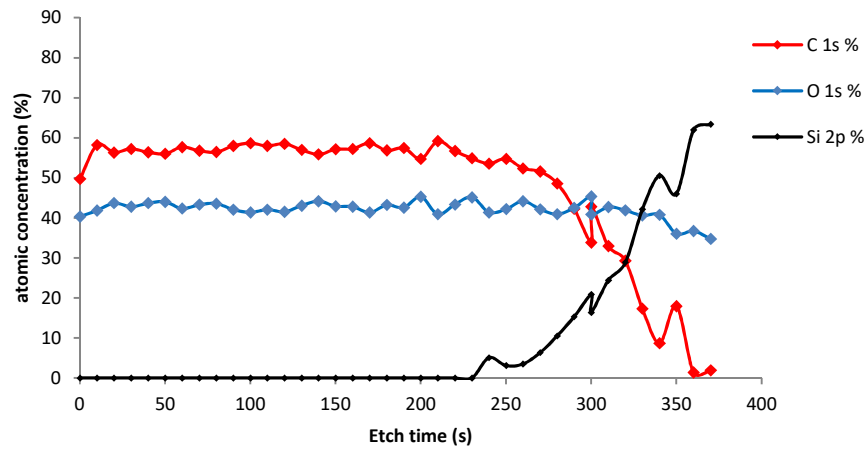
Carbon environments are shown in red, whilst oxygen environments are shown in blue. O/C = 0.8

The through thickness depth profile in *Figure 36a* details the change in atomic concentrations of carbon, oxygen and silicon. Plotting the oxygen to carbon ratio allows the analyst to compare the measured ratio against the theoretical or calculated ratio, determining the presence of excess oxygen or carbon which may signify addition sources of oxygen or carbon in the material.

A steady state C1s/O1s ratio is observed in the profile until the silicon wafer/PLGA interface is reached at 220s. Plotting of the theoretical O/C ratio based on the carbon oxygen environments with the measured O/C ratio is shown in *Figure 36b*.

The calculated O/C ratio was determined to be 0.8, a literature source for XPS analysis of PLGA films by A. Shibata et al¹⁴⁸ was also plotted with a given O/C ratio of 0.785. C1s and Si2p measured atomic concentration is also shown to identify the interface between film and substrate in the profile. The measured O/C ratio equals the calculated ratio until 250s etch where the ratio increases as the silicon wafer substrate is reached.

a)



b)

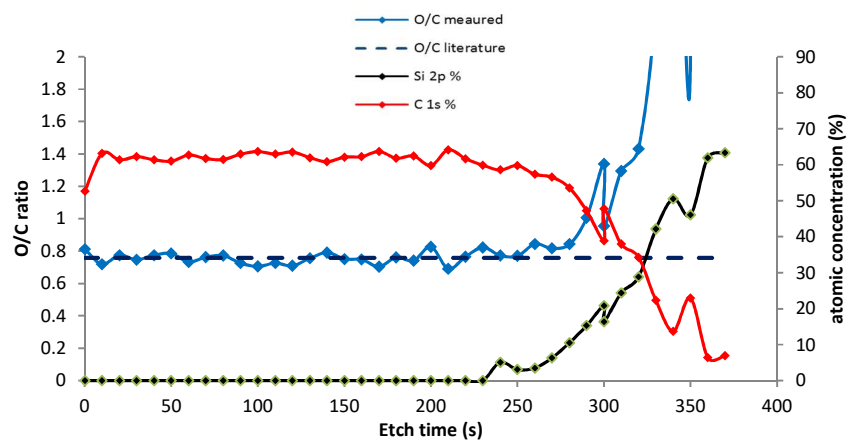


Figure 36 – XPS Ar_{1200} 5keV depth profile of PLGA film.

A) Monitoring changes in atomic concentration of oxygen, carbon and silicon in Ar_{1200} depth profile, b) O/C ratio change through PLGA film, measured and theoretic O/C ratio is shown on the left y axis, whilst atomic concentration is plotted on the right to show silicon wafer interface.

Establishing that quantitative elemental information could be acquired in 3D in the reference PLGA film allowed us to move onto the more complex hydrated pHEMA hydrogel. The developed preparatory procedure for analysis of pHEMA hydrogels by ToF-SIMS required using an inert gas to evacuate the surface post plunge freezing to remove frost condensed on the sample. This was performed in the same manner when visible frosting had occurred post snap freezing pHEMA hydrogel films for XPS, however if no frosting was observed on the sample from plunge freezing and the resultant sample transfer most likely due to the higher thermal conductivity of the copper stub resulting in a lower sample temperature as opposed to mounting on the ToF-SIMS cryostage. Entry to the precooled -130°C XPS entry chamber was performed without the need for argon gas evacuation. The sample transfer time from liquid nitrogen immersion to the entry chamber was performed rapidly as possible otherwise the accumulation of frost on the sample surface occurred when the sample was exposed to the ambient atmosphere. A description of the method of sample loading is further discussed below.

As we have seen through ToF-SIMS the entry of a hydrogel into a ultra-high vacuum is complex. A number of sample mountings were explored as unlike ToF-SIMS, the whole sample stage may not be plunged into the cryogenic liquid for snap freezing, rather the hydrogel must be mounted and pinned to a sample stub. This may be seen in **Figure 37**. Mounting of the hydrogel on silicon on a widely used XPS gold stub with steel pin resulted in frosted sample surface in the entry chamber and crystallisation of ice on the pHEMA surface. It was thought that the

thermal conductivity of the steel pin on the sample study may have been preventing cooling of the sample through thermal transfer causing an increase in sample temperature resulting in sublimation. The cold finger on the entry chamber that cools the sample stage in ToF-SIMS is comprised on a copper sheet. The thermal conductivity of copper as compared to steel is 10 times greater (350 W/m K at 20°C). Replacing the steel sample sub as evidenced in **Figure 37** prevented the crystallisation of ice on the sample surface and a suitably frozen-hydrated sample. The same argon air gas evacuation of the sample surface was employed in the XPS study if the sample showed significant condensation of frost on the sample surface. We found that transfer time c.a. 5 seconds on the sample stub was sufficient to prevent frost condensation on the sample surface upon loading the sample to the entry chamber in XPS, therefore air gun removal was not employed. This presents a difference in preparatory procedure, however we established that sample viability may be aided by air gun assisted removal of frost, if that occurs.

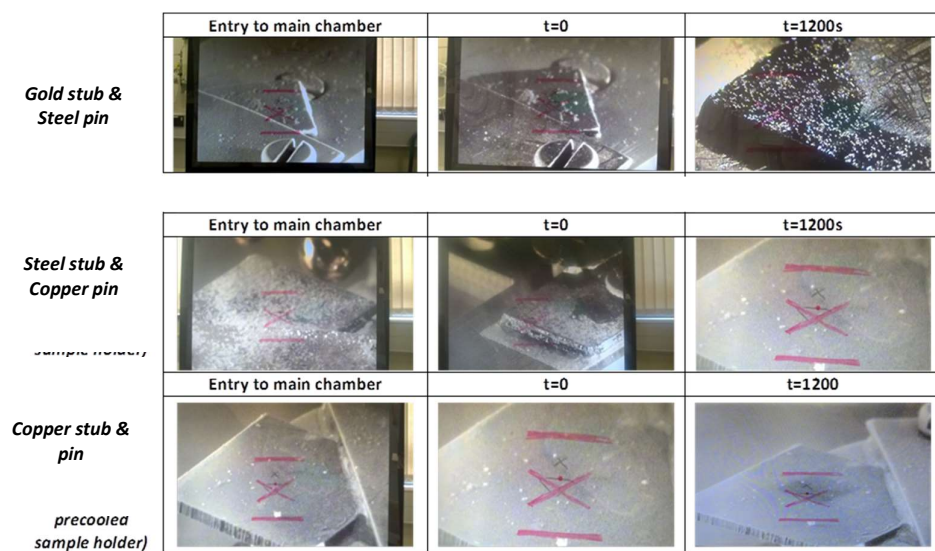


Figure 37 – Preparatory steps for introduction of frozen-hydrated pHEMA hydrogel samples for XPS analysis. Images of the sample after entry to analysis chamber, before etching and after 1200 seconds of argon etching are shown.

The data from low temperature analysis of a pHEMA film in a frozen hydrated hydrogel is now discussed. The structure of pHEMA is shown in **Figure 38** where 3 distinct oxygen and 6 carbon environments are shown. There are 2 different chemical environments for oxygen O-C and O=C, and 4 different environments for carbon: C-C, C-O, -CC-C(=O)OX and C(=O)OX.

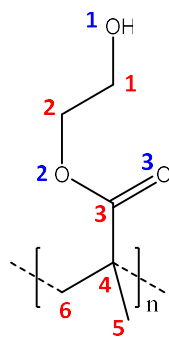


Figure 38 - Chemical structure of pHEMA showing oxygen and carbon environments.

Carbon environments are shown in red, whilst oxygen are shown in blue

The widescan spectra of the surface in **Figure 39a** show the presence of oxygen and carbon, where the O1s peak decreases in the 1500 s etch spectra as the bulk pHEMA film is analysed. After 2200 seconds a silicon 2p peak is observed indicating that the silicon substrate has been reached.

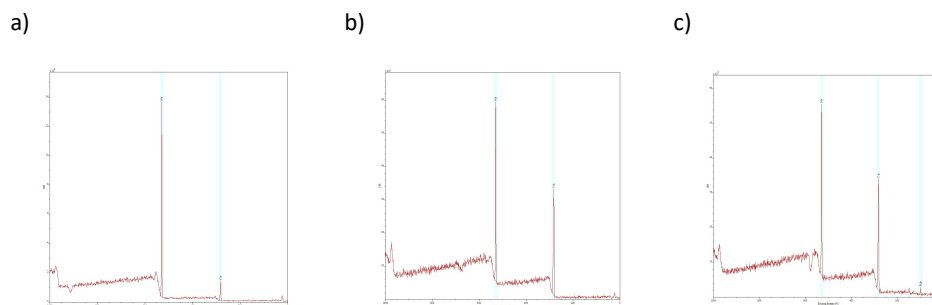


Figure 39 – Widescan XPS spectra from various etch times of a pHEMA film.

a) 0s etch, b) 1500s and c) 2200s

The respective 3D profile of the pHEMA hydrogel is shown in *Figure 40a*. The concentration of oxygen at the surface region is higher compared to the bulk, which decreases to a steady state concentration after 1000s etching conversely to carbon, where the amount of carbon at the surface is low before increasing to a steady state at 1000s. The concentration of carbon and oxygen decreases as the silicon wafer substrate is reached at 2000s.

Figure 40b shows the relationship between the oxygen/carbon ratio and etch time. The ratio is higher at the beginning of the profile, and decreases to the level of the calculated (0.5) and previously reported O/C ratio (0.498) from literature¹⁴⁹. Using the theoretical O/C ratio allows the determination of the hydration level of the pHEMA film which is shown in *Figure 40b*. pure pHEMA has an oxygen atomic percentage of 33%. An oxygen percentage above which must be contributed from an additional source. Any additional source of oxygen from this experiment will be from frozen water or the oxide layer on the silicon wafer. Subtracting the atomic concentration of oxygen in pure pHEMA in literature from the measured oxygen atomic concentration in the depth profile of the frozen pHEMA allows us to show the percentage of ice in the film. The percentage of ice at the surface of the film is around 40% which decreases to 0% after 1000s etching, indicating no water ice is present in the pHEMA film at this point.

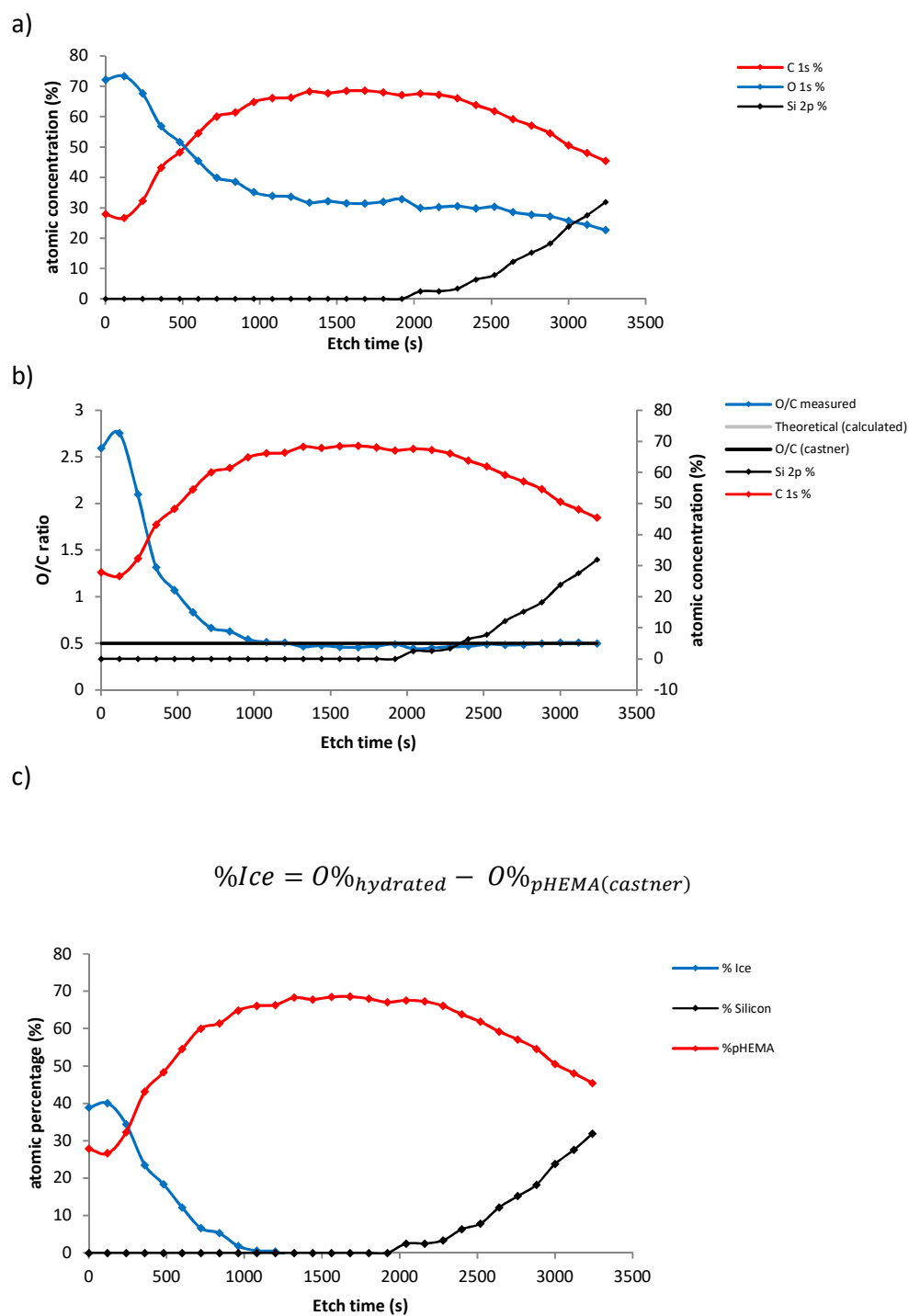


Figure 40 - Ar1200 5kev depth profile of phema film with calculation for percentage of ice

a) Monitoring changes in atomic concentration of oxygen, carbon and silicon in Ar₁₂₀₀ depth profile, b) O/C ratio change through phema film, measured and theoretic O/C ratio is shown on the left y axis, whilst atomic concentration of carbon and silicon is plotted on the right to show interfacial regions. c) Percentage of ice in the film is shown through the pHEMA film.

XPS depth profiling of the lysozyme incubated pHEMA hydrogel is shown in Error! Reference source not found. - **Figure 43**. Profilometry of the film prior to etching was performed to calibrate etch time to film thickness (**Figure 41**), which resulted in a film thickness of c.a. 4.2 μm .

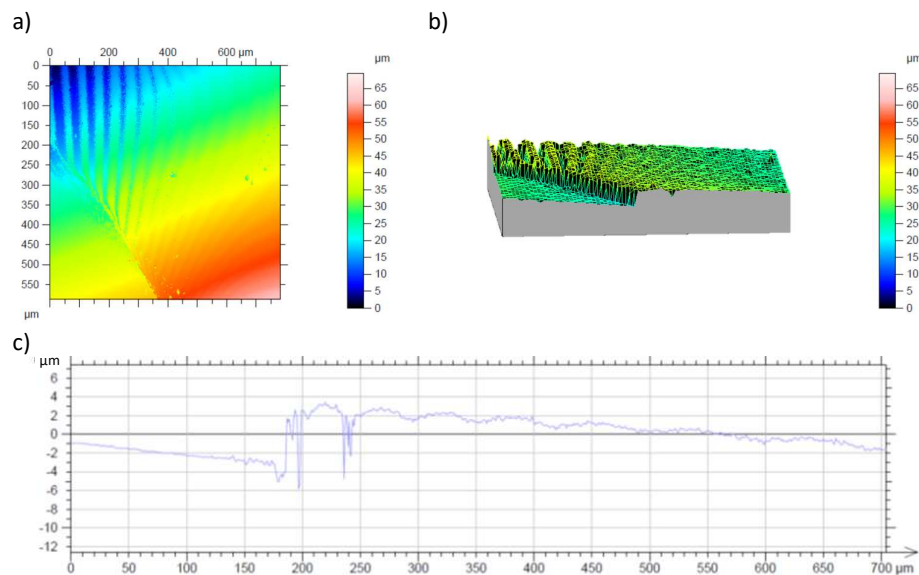


Figure 41 – Surface profilometry of pHEMA film produced by 1 dip in 6 wt% pHEMA solution.

a) 2D profile, b) 3D cross section, c) thickness extraction profile of pHEMA film

XPS analysis of the surface shown in Error! Reference source not found. indicates the presence of nitrogen, oxygen and carbon. After 1500 seconds etching the amount of nitrogen decreases and a higher oxygen/carbon ratio is observed. After 2200 seconds etch silicon 2p peak appears indicating the substrate has been reached.

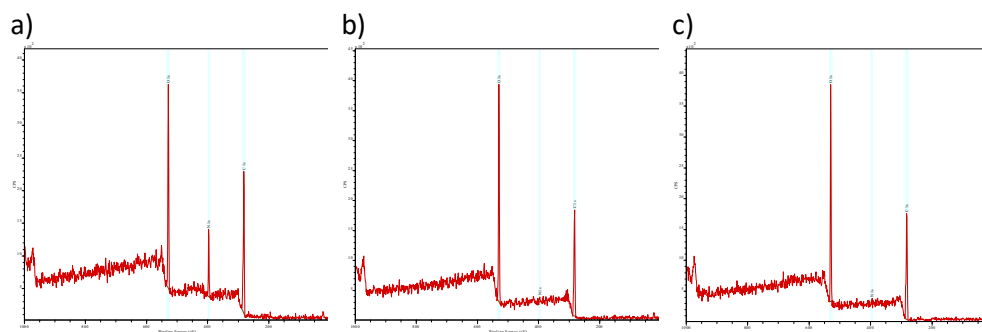


Figure 42 - Widescan spectra from various etch times in PHEMA film.

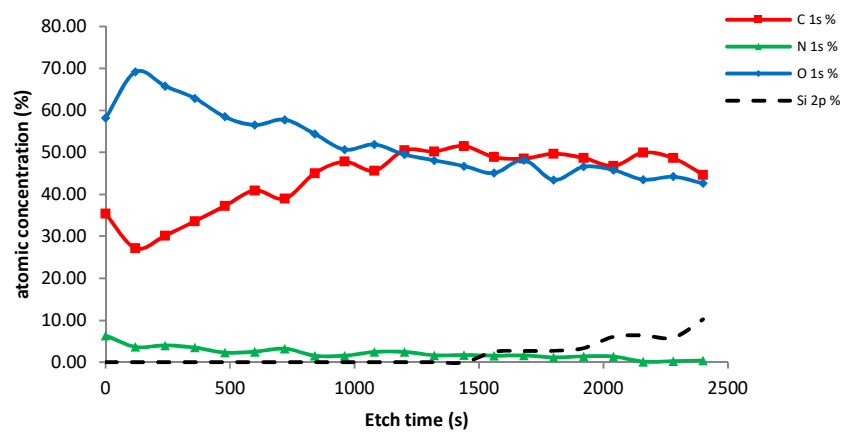
a) 0s etch, b) 1500s and c) 2200s

The through thickness profile in **Figure 43** is the first of 2 repeat profiles acquired for this sample format. A higher concentration of oxygen is present at the surface as compared to the bulk film which decreases to a steady state concentration to 50% after 1000 seconds etching, the same is observed of carbon which has a lowered concentration at the surface, and increases to a steady state at 1000s. The nitrogen concentration is higher at the surface, and decreases gradually through the hydrogel until the interface is reached at 1500 seconds etching. The carbon and oxygen concentration remains at a steady state concentration from 1000s for the rest of the profile.

The repeat in *Figure 43* is similar as a higher surface concentration is present for oxygen and lowered for carbon. A steady state region is observed from 1800 seconds to 2500 seconds etch.

Conversion of etch time to depth was performed to assess the ingress of lysozyme as a function of film thickness. Profilometry of the dehydrated film before incubation in the lysozyme solution indicated the film thickness to be $4.2\ \mu\text{m}$ (*Figure 41*). The calculated interface at 50% C1s intensity was used to estimate the interface between ice and pHEMA and silicon wafer and pHEMA then calibrate to the measured film thickness. The conversion to thickness is shown in *Figure 44a/b* where both repeats are plotted against film thickness. The error for nitrogen 1s atomic concentration is within 2% for the combined profiles. The sample size is relatively small at 2 however the low error suggests repeatability in measurement.

a)



b)

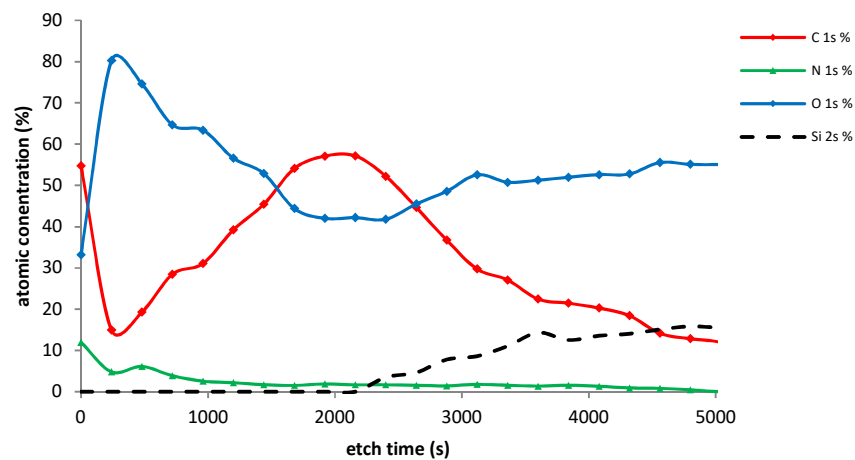
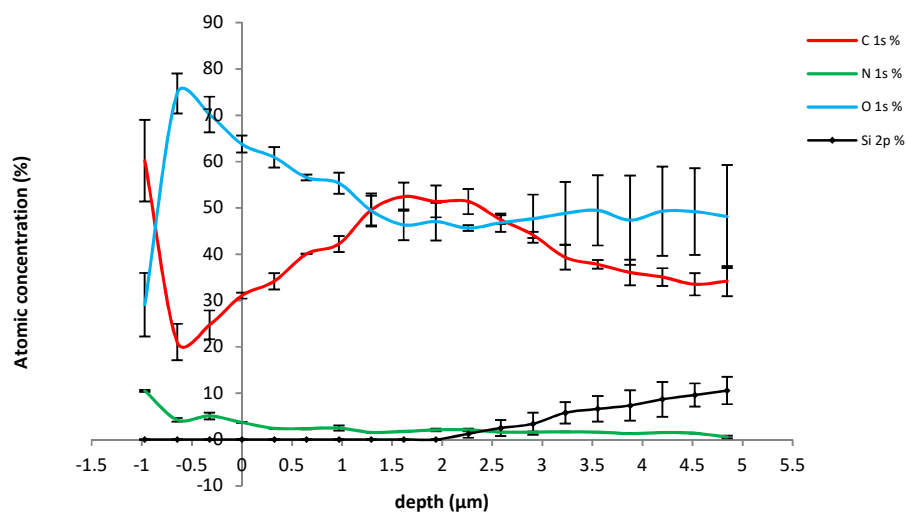


Figure 43 - Ar_{1200} 5keV depth profile of pHEMA film incubated in 1% lysozyme solution.

a/b) Repeat depth profiles monitoring changes in atomic concentration of oxygen, carbon and silicon in Ar_{1200} depth profile, b) Changes in atomic concentration of oxygen (blue), carbon (red), nitrogen (green) and silicon (black dash) as a function of etch time through pHEMA film incubated in lysozyme solution

a)



b)

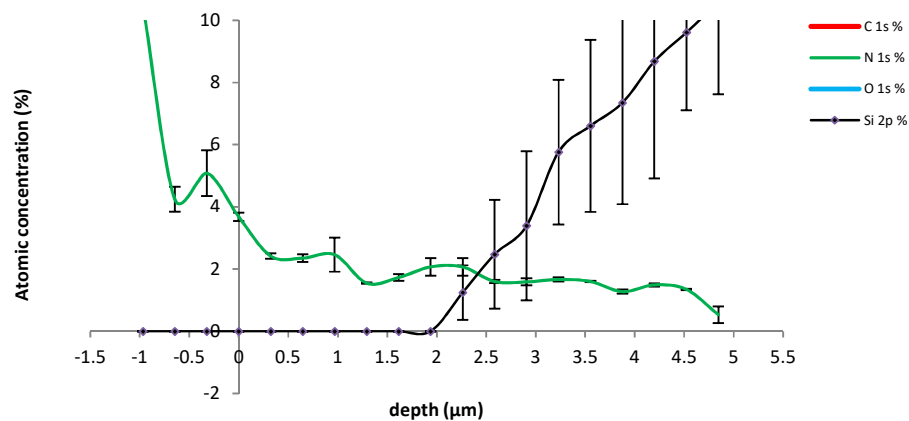


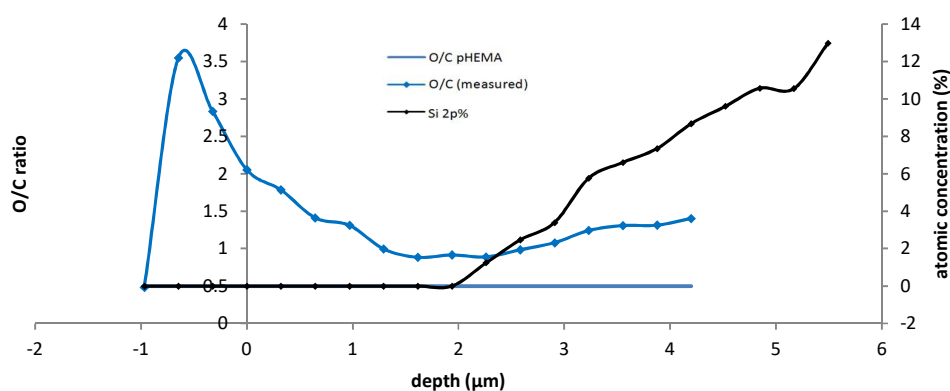
Figure 44 - Ar_{1200} 5keV depth profile of pHEMA film incubated in 1% lysozyme solution.

a) pHEMA film with lysozyme incubated in 1% lysozyme solution, where error bars based on error percentage have been plotted for $n=2$. b) expanded x axis to show lysozyme distribution plotted in green

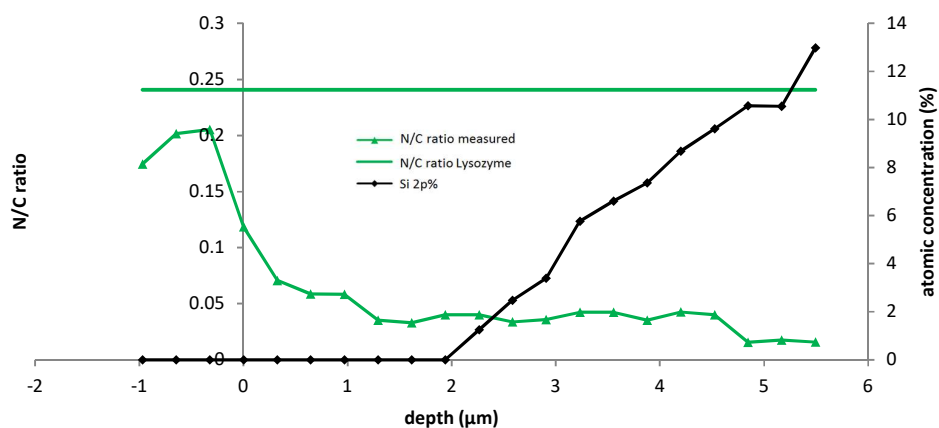
Plotting the ratio of oxygen to carbon allows us to assess if the pHEMA hydrogel reaches an O/C ratio that reflects dehydrated pHEMA (0.5), the plot of which is shown in **Figure 45a**. The ratio is higher at the surface and decreases for subsequent analysis cycles into the bulk pHEMA film to a minimum of 1 at 1.5 μm . The O/C ratio increases at the interface region between silicon wafer and pHEMA as the oxide layer on the silicon substrate is analysed, contributing to the oxygen atomic concentration at this point in the profile.

The measured N/C ratio through the hydrogel film is shown in **Figure 45b**. The ratio is high in the surface region, 0.2 and decreases to 0.05 in the bulk film, as more carbon is present in the film from pHEMA, as composed to the interface region. The measured N/O ratio through the hydrogel film is shown in **Figure 45c**. The ratio is for the initial analysis pre etching (0.4) and decreases to minimum in the bulk film (0.05), until the interface is reached at 4.2 μm .

a)



b)



d)

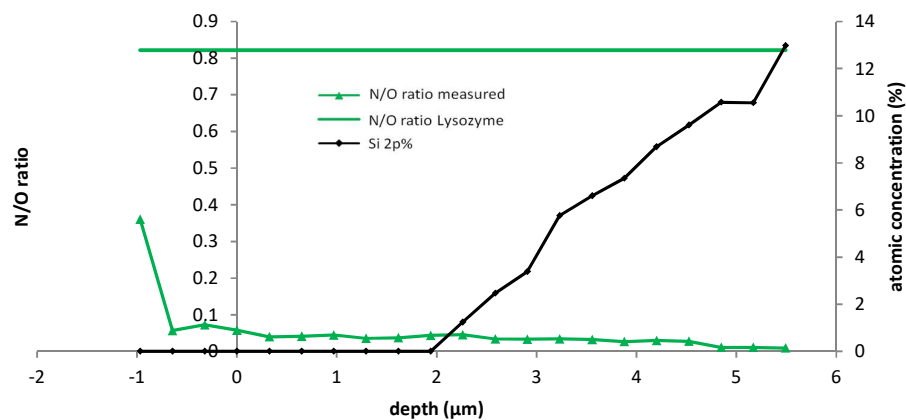


Figure 45 - Nitrogen change as a function of depth through pHEMA/lysozyme hydrogel

a) Measuring O/C ratio change through pHEMA/lysozyme hydrogel, O/C ratio acquired from literature for pure lysozyme is plotted against measured, atomic concentration is shown on the right y-axis to indicate silicon interface. b) N/C ratio change through pHEMA/lysozyme hydrogel, n/c ratio for lysozyme (literature) is plotted to indicate 100% lysozyme. d) N/O ratio change through pHEMA/lysozyme hydrogel, N/O ratio for lysozyme (literature) is plotted to indicate 100%

The contribution of lysozyme and pHEMA to the percentage of carbon present in the film may be calculated from the N/C ratio of pure lysozyme and the percentage of nitrogen in the film. This gives an idea on the relative amount of lysozyme within the film. An example calculation is shown below in **Table 11** and the resultant depth profile of the contributions from pHEMA and Lysozyme is shown in **Figure 46**. The percentage of carbon attributed to lysozyme at the surface is high at the surface region at around 45% as compared to in the bulk at 10%. The percentage of carbon contributed from pHEMA at the surface is around 15% whereas the percentage of carbon from pHEMA in the bulk film is higher at 45-40%.

Table 11 – contribution of lysozyme and pHEMA to carbon concentration in depth profile

Calculation	Example (1 st data point)
$\frac{N\%_{Lys}}{C\%_{Lys}} = N/C_{Lys}$	$\frac{15.7}{65.2} = 0.2428$
$(N\%) \times N/C_{Lys} = \% C_{Lys}$	$10.5 \times 0.2428 = 43.8 \%$
$(\% C) - (\% C_{lys}) = \% C_{pHEMA}$	$60.2 - 43.8 = 16.4$

a)

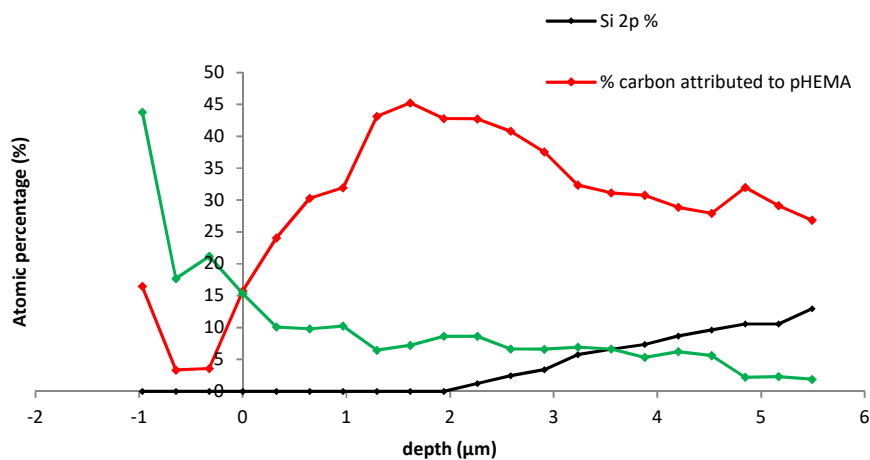


Figure 46 – Atomic percentage of carbon in film contributed by lysozyme and pHEMA

We attempted to correlate depth profiling ToF-SIMS data of the same sample thickness (c.a. 4.2 μm) pHEMA film which is shown in *Figure 47a* and *Figure 47b*.

Previously we had established that argon gas may be used to clear the sample surface from frosting under transfer from the cryogen to the pre-cooled entry chamber. The Initial experiments on pHEMA measured a sample transfer time of 8 seconds. The sample time for loading was able to be minimised by 3 seconds by the aid of additional personnel to open and pump down the entry chamber once closed whilst the principal investigator loaded the cooled cryostages. The longer sample loading time in the initial experiments for ToF-SIMS, identified that significant surface frosting of the sample was minimal from 5-seconds exposure of the frozen-hydrated sample to the ambient atmosphere (19°C). A sample transfer time of 5 seconds was used in both of the ToF-SIMS and XPS depth profiling experiments in 5.2 and 5.3 therefore are comparable.

For both the profiles shown in *Figure 47a* and *Figure 47b* differential thickness of ice was present on the surface. This is evidenced by the different etch times (530/450 seconds) taken to reach the interfacial region between pHEMA film and ice. This was corrected for by assuming an interface at 50% $\text{C}_6\text{H}_9\text{O}_2^+$ max intensity at the interfacial regions between ice and pHEMA, and pHEMA and silicon wafer. Calibrating the profiles to a dehydrated thickness of ca. 4.2 μm to the interface position between pHEMA and silicon wafer was performed, measured though White-light Interferometry. The thickness at 0 μm was assessed at the 50% max intensity of $\text{C}_6\text{H}_9\text{O}_2^+$ at the interface between ice and pHEMA hydrogel.

Both profiles in **Figure 47a** and **Figure 47b** are similar. A stable $\text{C}_6\text{H}_9\text{O}_2^+$ ion yield in the steady state region is observed in both. The intensity of the polyatomic water cluster signifying ice is high in the ice film and decreases to a background intensity at the interface between silicon wafer and gel in both profiles. The deviation between the two repeats is shown in **Figure 47c**. The $\text{C}_6\text{H}_9\text{O}_2^+$ electropositive secondary ion intensity shows a low standard deviation at the ice/gel interface and steady state region, conversely a large deviation is observed at the interface between gel and silicon wafer. It should be noted that the sample size $n=2$ in this study is low. The expanded y-scale showing $\text{C}_4\text{H}_6\text{N}_2^+$ distribution for the two repeats (**Figure 48a**) indicates that a reduced amount of lysozyme is present in the vitreous ice layer as compared to the bulk gel. The signal intensity of $\text{C}_4\text{H}_6\text{N}_2^+$ in the pHEMA film decreases to 0.001 at the interface between pHEMA and silicon from a maximum of 0.0015. The difference in measured ion intensity between the two in this small sample size suggests that lysozyme distribution is the same for both samples, indicating repeatability. In **Figure 48** $\text{C}_4\text{H}_6\text{N}_2^+$ normalised intensities and nitrogen atomic concentrations from depth profiling the same sample type by ToF-SIMS and XPS are displayed as a function of film depth. The intercept on the x-axis indicates the gel interface at ca. 50% $\text{C}_6\text{H}_9\text{O}_2^+$ intensity, the negative units on the x-scale representing the thickness of ice at the gel surface plotted in the profile. $\text{C}_4\text{H}_6\text{N}_2^+$ ion intensity is reduced at the surface region, and increases, in the bulk of the gel, degrading towards the silicon wafer/film interface. Contrastingly the atomic concentration of nitrogen is elevated at the surface, whereas decreases in the bulk, degrading similarly to $\text{C}_4\text{H}_6\text{N}_2^+$ normalised intensity at the silicon wafer/film interface.

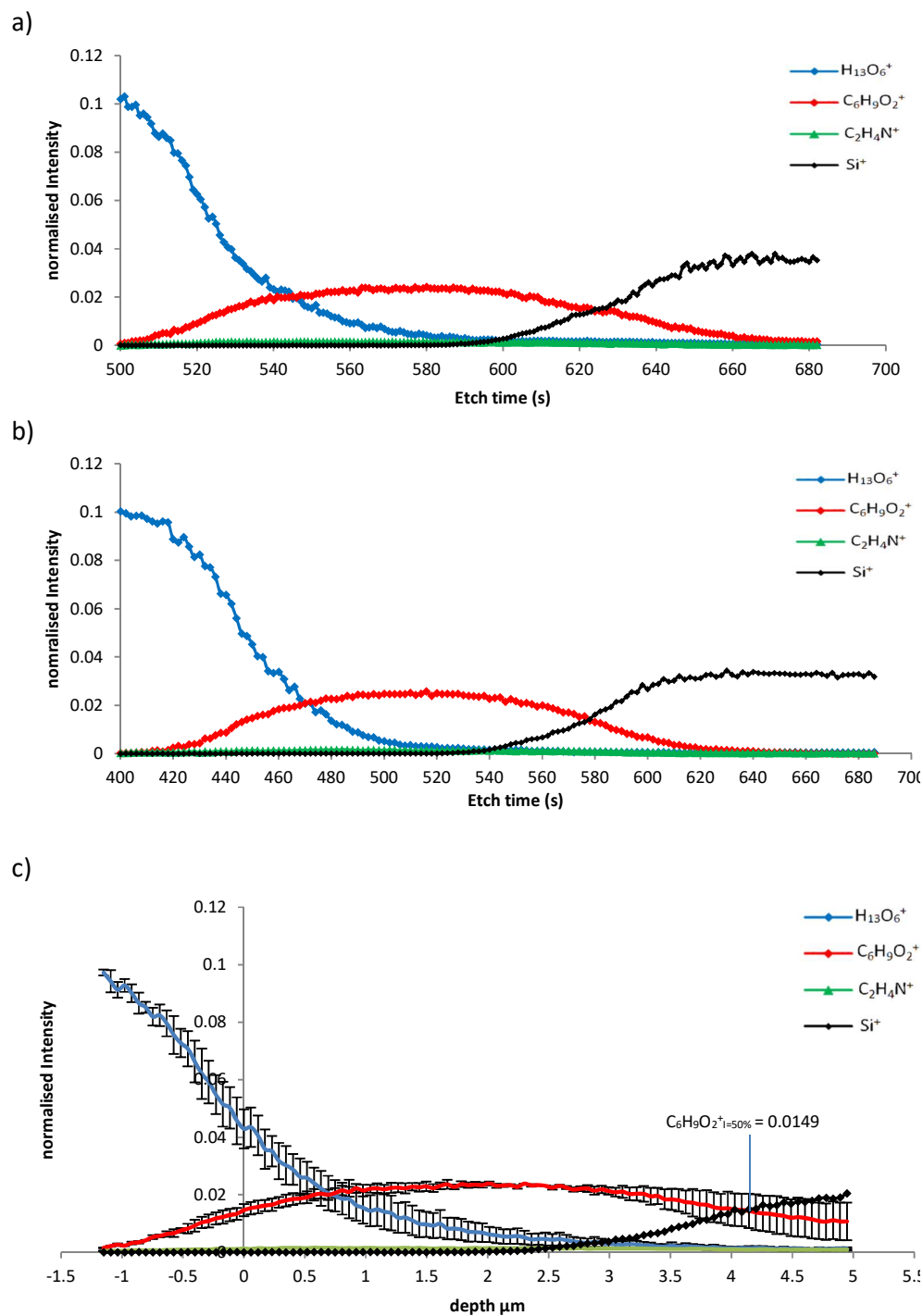
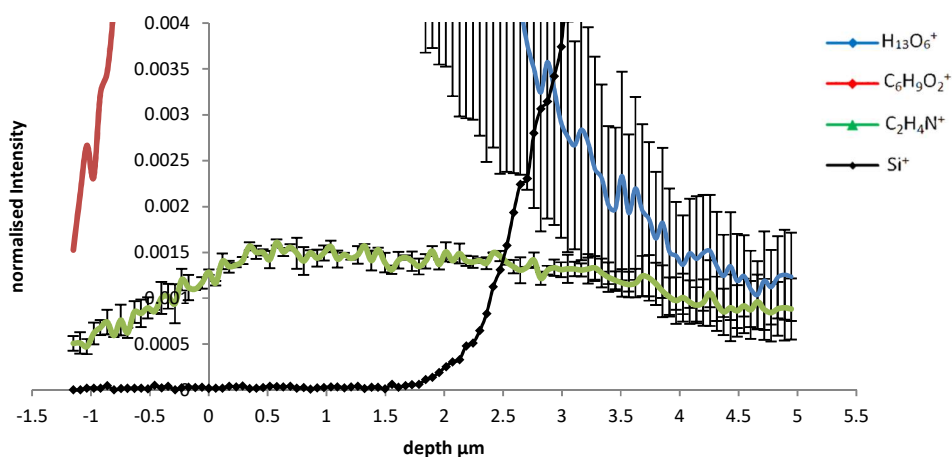


Figure 47 - ToF-SIMS depth profile of lysozyme incubated hydrogel

a,b) ToF-SIMS depth profiles of pHEMA hydrogels incubated in water/lysozyme for 16 hours at 20°C. c) Error plotted as standard deviation between the two samples ice/pHEMA interface at 0 microns, silicon wafer/pHEMA interface at 4.2 μm.

a)



b)

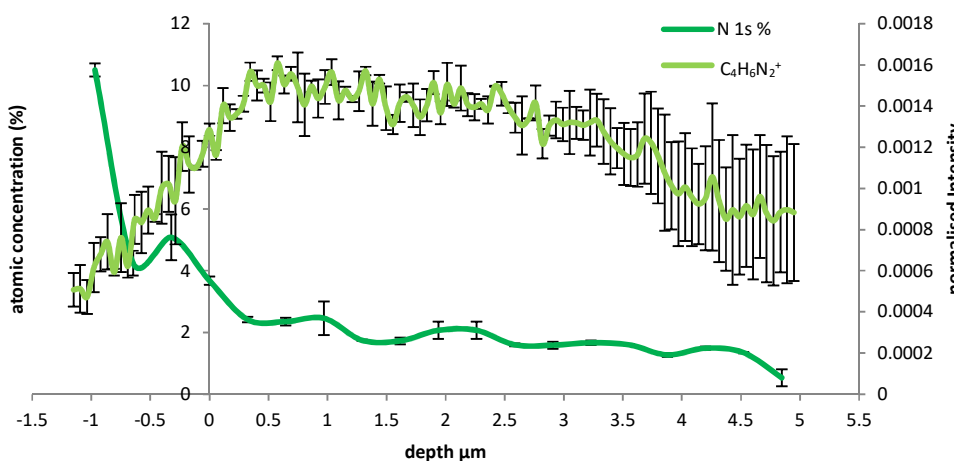


Figure 48 - ToF-SIMS/XPS depth profile of lysozyme incubated hydrogel

a) expanded y-axis in TOF-SIMS argon depth profile of hydrated pHEMA film, $\text{C}_4\text{H}_6\text{N}_2^+$ represents lysozyme (green), $\text{C}_6\text{H}_9\text{O}_2^+$ represents pHEMA (red), $\text{H}_{13}\text{O}_6^+$ represents ice as a polyatomic water cluster, and Si^+ represents silicon wafer. b) comparison between the measured concentration of lysozyme represented by N 1s (XPS) and $\text{C}_4\text{H}_6\text{N}_2^+$ (ToF-SIMS)

Our ToF-SIMS analyses of pHEMA gave us confidence that mapping lysozyme distribution within pHEMA hydrogel in frozen hydrated format was possible. The matrix effect is however problematic, as the measured changes in secondary ion intensities may be strongly influenced by the electronic state of the material. For this reason, correlating XPS data on nitrogen/carbon changes in the pHEMA hydrogel allows us to determine if there is an observable difference between the two parallel techniques in terms of $C_4H_6N_2^+$ secondary ion intensity and N/C ratio. Plotting $C_4H_6N_2^+$ normalised intensity with N1s atomic concentration from XPS allows us to assess if this gradient distribution of lysozyme is observed similarly to ToF-SIMS. Differences in the interfacial ice/pHEMA region between XPS and ToF-SIMS are observed. The concentration of nitrogen increases in the surface region of *Figure 48b* observed in XPS whilst $C_4H_6N_2^+$ normalised intensity decreases in this region in the ToF-SIMS depth profile. *Figure 49a/b* displays the relationship between $C_2H_6N_2^+$ and Nitrogen concentration. Plotting the relationship between XPS and ToF-SIMS allows us to assess if the relationship between ion intensity and concentration of lysozyme is linear. The data points from 0.5 – 4.5 μm depth analysis were plotted as significant matrix effect was observed in the first 0.5 microns of analysis at the ice/gel interface. The relationship appears to be relatively linear, where an increase in the N/C ratio/molar concentration of lysozyme correlates to a higher $C_4H_6N_2^+$ ion intensity.

a)

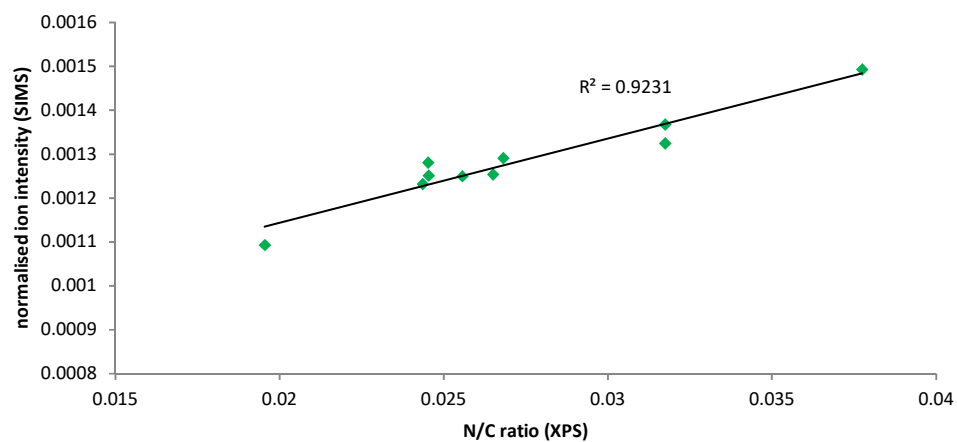


Figure 49 - Determining linearity between $C_4H_6N_2^+$ (ToF-SIMS) intensities and nitrogen concentration (xps)

suppression of $C_4H_6N_2^+$ secondary ion intensity observed in the ice film in ToF-SIMS, therefore the surface region was negated in xps/tof-sims plot. a) ratio of nitrogen to carbon against the normalised $C_4H_6N_2^+$ intensity through profile.

5.4. DISCUSSION

This study aimed to apply a simple sample preparation procedure for ToF-SIMS to achieve a frozen hydrated hydrogel for analysis without dry box sample entry facilities and demonstrate the ability to determine biomolecule distribution in a frozen hydrated format. The method is anticipated to be equally applicable to other vacuum based analytical techniques. We determined that water was retained in all samples by detection of $\text{H}(\text{H}_2\text{O})_n^+$ secondary ions signifying a frozen hydrated state. Previous studies have reported ToF-SIMS molecular depth profiling of ice indicated by the detection of these clusters in a histadine/ice film^{134,150}. The spectra from depth profiling each of the three hydrogel sample compositions shows evidence of water cluster secondary ions beginning at $n = 5$ ($m/z = 91.688$) to $n = 11$ ($m/z = 199.130$), which shows a similarity to the spectra observed previously for pure ice films¹³⁴.

The following low temperature analysis of each of the sample formats was effective to produce a full through-thickness chemical map. This method has been employed across 3 differing sample formats showing the versatility of the preparatory procedure in high vacuum frozen hydrated hydrogel analysis. This study aimed to characterise biomolecule distribution within hydrogels using the method developed.

A model biomolecule was used, lysozyme, and incorporated within the hydrogel either by diffusion into the pHEMA film during incubation in an aqueous solution or by mixing with the polymer with the pHEMA solution prior to depositing the film. In both cases lysozyme was detected throughout the film. Using the relative $\text{C}_4\text{H}_6\text{N}_2^+$ ion intensities within a sample of constant water and polymer content is

thought to be valid to characterise the protein distribution as a function of depth, as the $C_4H_6N_2^+$ ion peak was only seen in samples contain lysozyme while was absent in the pure pHEMA sample. When comparing between samples of differing composition the mass spectrometry matrix effect needs to be considered as this may dramatically affect secondary ion yields. The most marked difference in secondary ion intensities is the decrease of the both the characteristic polymer ($C_6H_9O_2^+$) and the water ($H_{13}O_6^+$) intensity of upon lysozyme incorporation (*Figure 33*). This suggests that the change in chemistry of the sample associated with the introduction of lysozyme suppresses secondary ion emission (*Table 10*) since we know that the molar reduction in the constituents (0.06 % pHEMA) could not account for the near 10 fold decrease in $C_6H_9O_2^+$ ion intensity from *Figure 33a to c*. Quantitative comparisons between pHEMA hydrogel samples is therefore not readily possible, however qualitatively comparing lysozyme distribution and thus mobility is possible in pHEMA hydrogels.

An enriched lysozyme surface layer is observed when lysozyme is combined as a component of the dip coating solution signifying that lysozyme diffuses towards the hydrogel–water interface, presumably as a result of leaching into the water. In the pure pHEMA sample, complementary pHEMA depletion and water enrichment is indicated by the characteristic ion trends with etch time (*Figure 33a*). Such complementary and self-consistent trends are not seen in from the samples with lysozyme (*Figure 33B/C*) suggesting that this incorporation induces ‘surface transience’ supporting the view of a significant role for the matrix effect related to lysozyme presence. Recent studies using ToF-SIMS depth profiling illustrate the

complexity of this effect when depth profiling organic materials which has found to dramatically inhibit secondary ion intensities of films comprised on organic compounds¹⁵¹.

This following section describes the chemical characterisation a frozen hydrated pHEMA hydrogel by XPS, the first example of depth profiling XPS of this sample type.

The majority of studies to date have been limited to the surface of freeze dried hydrogels by either freeze fracture or freeze drying when entered into vacuum. Our approach employing an Ar-GCIB as an etching source allows the mapping of protein distribution in a chemical state reflecting hydration. We determined that the developed preparatory procedure for the analysis of frozen-hydrated pHEMA hydrogels was appropriate to retain water in the hydrogel. This may be evidenced by the excess oxygen concentration in the pHEMA films which is contributed by ice within the hydrogel.

Similarly to the ToF-SIMS study, we aimed to analyse the distribution of lysozyme, in aid to expand ToF-SIMS quantitatively for hydrogel analysis. This allows us to provide quantitative molecular information on protein distribution in hydrogels.

PLGA provided stable depth profiles where the amount of oxygen and carbon in the film reflected the theoretical ratio of 0.8 acquired from literature and calculated. The following low temperature analysis of each of the pHEMA sample formats was effective to produce a full through-thickness chemical map. The initial analysis of the pHEMA gel in the absence of lysozyme was performed as the introduction of lysozyme adds an additional component into the sample

presenting complexity in quantification an lysozyme contains a mixture of organic components, whilst an excess of oxygen in the pHEMA hydrogel may only be contributed from ice, and any carbon may be associated with pHEMA or carbonaceous contamination.

The theoretical O/C ratio of pHEMA has been well reported in literature¹³³. This allowed us to assess gel dehydration as a reduction below the ratio of 1:2 oxygens to carbons. The O/C carbon ratio in the frozen-hydrated pHEMA hydrogel in the absence of lysozyme, is dehydrated in the bulk film, with O/C ratio equalling pure pHEMA (*Figure 40*). The rate of mass transfer from water in the solid phase or vacuum induced sublimation will be greater at the air-pHEMA interface, as the sample is cooled from the cold-finger attachment through the sample stage. Sublimation of ice should be higher in the surface region, as compared to the bulk film. The oxygen concentration however decreases the further the film is profiled, suggesting that the film becomes increasingly dehydrated. Subsequent etching cycles produce more dehydration, therefore it may be a result of Argon bombardment and subsequent analysis of the etch crater that decreases the amount of ice in the top 10nm of the surface analysed.

For the pHEMA films immersed in lysozyme solution (*Figure 43*), lysozyme diffuses into the pHEMA film a gradient distribution. This is observed from the surface to the 1st micron of the gel as a higher concentration of nitrogen is present in the ice layer at the surface as compared to the pHEMA film, suggesting that the lysozyme is mobile within the film.

We aimed to demonstrate that lysozyme distribution in pHEMA hydrogels could be quantitatively determined using ToF-SIMS. The differences between the analysis conditions of XPS and ToF-SIMS may be minimised to limit the experimental error associated with this such as ensuring the same sputter beam energy of 10 KeV at a current of 9 nA was used in both analyses. There are however differences in the operational mode which may impact the quality of data between the two techniques. The registered temperature in the analysis chamber was held at is around 50°C cooler at -160°C than the analysis conditions under ToF-SIMS. In hydrated measurement the sample temperature will ideally be as cold as possible to prevent hexagonal ice formation within the hydrogel under vacuum. The rate of ice sublimation under vacuum is strongly affected by the sample temperature^{107–109}, meaning sublimation rate is higher in warmer samples. The vacuum pressure in XPS analysis as compared to ToF-SIMS is an order of magnitude higher at 6.6×10^{-8} mbar compared to 6×10^{-7} mbar in ToF-SIMS; it is therefore plausible that the reduced pressure may be resulting in promotion of sublimation. Kossacki et al¹¹⁰ demonstrated this on demineralised and mineralised ice films, showing sublimation dramatically increased above 200 Kelvin at 5×10^{-5} mbar, other examples in literature are also reported^{102,111,112}.

When assessing lysozyme distribution as function of depth through SIMS, the lowered concentration at the ice/gel surface interface is interesting, as it shows a trend opposite to that observed for nitrogen in XPS. It is to be expected that a higher concentration should be present at the surface region as the concentration of lysozyme in the incubation medium should higher than in the pHEMA film,

however we see a reduction in ion intensity in this region. If we assume that the freezing process and subsequent entry to the analysis chamber does not sufficiently affect the lysozyme distribution in the vitreous ice layer, the reduction in measured intensity therefore must be an electronic effect rather than an effect of redistribution. Aoki et al determined that small amounts of ice on peptide surfaces enhanced the ionisation yields high mass fragments such as angiotensin II (m/z 1046) and found that ionisation enhancement was only observed when 1 to 2 nm of ice was deposited, however when larger amount of ice is deposited on the sample surface, the desorption of the angiotensin II associated ions was inhibited¹⁵², which may explain why we see a reduction in $C_4H_6N_2^+$ intensity in the surface ice film.

Comparison of $C_4H_6N_2^+$ ion intensity and the molar concentration of lysozyme calculated from XPS data, shows the relationship between normalised Intensity and N/C ratio/lysozyme concentration is relatively linear evidenced by the R^2 value of 0.9231 giving evidence that ToF-SIMS data may be used independently to quantify Lysozyme distribution and amounts in frozen-hydrated pHEMA films.

5.5. CONCLUSION

The preparatory procedure developed in Chapter 4 has been proven to be suitable for introducing frozen-hydrated pHEMA/Lysozyme hydrogels into ToF-SIMS instruments.

Lysozyme was found to be sufficiently mobile within the hydrated hydrogel film to diffuse into the films from solution. When lysozyme is combined with pHEMA into the dip coating solution, it diffuses out of the hydrogel resulting in an increased loading at the surface.

A modified preparatory procedure was also suitable for introduction of frozen-hydrated pHEMA/lysozyme hydrogels into XPS instruments. The change in $C_4H_6N_2^+$ normalised intensities and C/N ratios in the profiles through ToF-SIMS and XPS were observed to be linear in the bulk of the gel film, however different at the ice/film interface as matrix effects were observed in this region suppressing $C_4H_6N_2^+$ intensity.

The two comparable thickness pHEMA films of 4.2 μm suggests that the films become increasingly dehydrated as a result of analysis condition in both ToF-SIMS and XPS.

CHAPTER 6 – STABILITY OF ACTIVATED SURFACES UNDER CRYO CONDITIONS

6.1. AIM

To assess the impact of using a gas cluster ion beam on the sample composition, moreover the response of hydrogels to cryo and UHV analysis conditions, a pHEMA/Lysozyme hydrogel film was continually analysed with no etching to determine the impact of static conditions through ToF-SIMS and XPS on the hydrogel chemical composition.

We show that condensation of water occurs on the sample surface of the pHEMA film evidenced by an increase in $\text{H}_{13}\text{O}_6^+$ peak intensity and oxygen concentration at the surface. We show that dehydration does not occur in pHEMA/Lysozyme films under static conditions, therefore the use of Argon gas clusters to sputter etch frozen-hydrated pHEMA films of 4.2 μm thickness results in increased reduction in the volume of frozen water in the film.

This is valuable information for XPS/ToF-SIMS users employing the frozen-hydrated format for 2D and 3D analysis of cells, tissues and hydrogels as ice sublimation may change the physical structure of the cell or film as a function of depth analysed.

6.2. INTRODUCTION

We have observed that significant sample changes are occurring in the frozen-hydrated pHEMA/lysozyme films under cryo conditions. More specifically that decreasing $\text{H}_{13}\text{O}_6^+$ intensity and oxygen percentage in ToF-SIMS and XPS respectively is occurring as the hydrogel is profiled. A steady state relationship should be observed if the hydrogel is maintaining the same hydration state through the entire film. This suggest that the hydrogel has either a decreasing volume of frozen water within the film, or that some part of the analysis conditions in both XPS and ToF-SIMS is causing the sample to dehydrate by sublimation of ice.

Surface based mass spectrometry in which an energetic probe (atomic ion, cluster ion, or laser) strikes surface catalyses the emission of non-volatile molecules for analysis. It is well established that increasing the impact energy of the projectile striking the surface will result in an increased sputter yield⁹⁴. As the energy of the projectile transfers into the sample surface, an increased ion beam current or voltage will result in a larger collision cascade and an increased ion yield compared to a lower beam current or voltage. This can be better observed through molecular simulations¹⁵³ of the sputtering process illustrated below in *Figure 50*.

The data so far suggests that a significant degree of sublimation of ice is occurring in the sample. Sublimation rate of ice is strongly dependent on analysis conditions such as sample temperature and pressure^{102,110–112}. The low vacuum environment is challenging in analysis of frozen-hydrated hydrogels as the physical state of the

hydrogel may be altered by the environment. More specifically vacuum induced sublimation of ice, freeze drying the sample.

Assessing the effect of analysis condition on the sample becomes more complex in depth profiling as the sample surface may be unique in physical and chemical composition at different depths in the hydrogel film. Previous experiments in ToF-SIMS depth profiling pHEMA hydrogels has shown us that a decreasing amount of water ice represented by $\text{H}_{13}\text{O}_6^+$ occurs through etching the pHEMA film. It is therefore logical that some part of the analysis procedure or conditions is causing this effect. It is reasonable to suggest that sputter etching with Ar-GCIB beam may result in energy transfer in the surface region which results in sublimation of ice in the analysis depth of the LMIG following sample etching. However, assessing the degree of sample change in static conditions allows us to decouple the effect of using Ar-GCIB analysis for frozen-hydrated 3D chemical analysis of hydrogels.

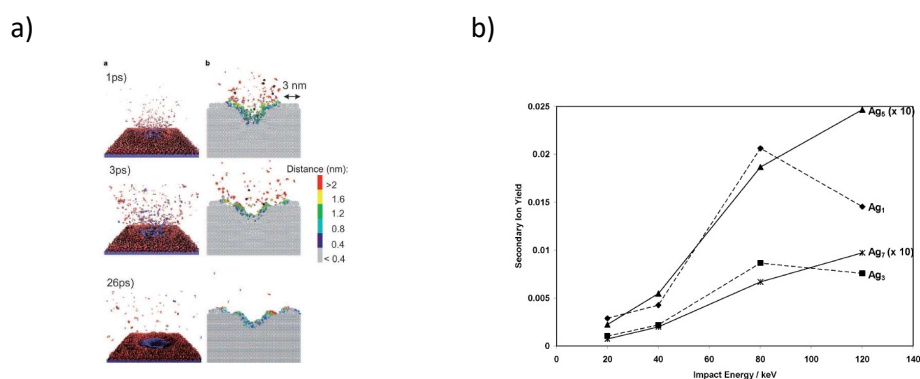


Figure 50 - a) 15 KeV C_{60} bombardment of benzene on Ag, the atomic positions at three time points of bombardment, b) plot of the secondary ion yield of the silver clusters for C_{60} impact energies of 20, 40, 80 and 120 KeV

J. S. Fletcher, X. A. Conlan, E. A. Jones, G. Biddulph, N. P. Lockyer, and J. C. Vickerman, 2006, 78, 1827–1831.

B. J. Garrison and Z. Postawa, *Mass Spectrom. Rev.*, 2008, 27, 289–315.

6.3. RESULTS

Assessing if chemical change in frozen-hydrated pHEMA films is occurring under static analysis conditions allowed us to determine if the observed sample dehydration in depth profiling was a factor of the hydrogel responding dynamic analysis conditions rather than static conditions.

De-coupling the impact of analysis conditions between static surface analysis and depth profiling is complex as depth profiling exposes a new area of the sample for each etch cycle, making comparison between the chemical states of the surfaces difficult. The amount of frozen water in hydrogel films under UHV conditions is dependent of the achievable vacuum pressure and the sample temperature, with lower vacuum pressures (6×10^{-8} mbar) and higher sample temperatures ($> -120^{\circ}\text{C}$) increasing the rate of ice sublimation whilst higher vacuum pressures (6×10^{-6} mbar) and lower sample temperatures ($< -120^{\circ}\text{C}$) reducing the rate of ice sublimation.

The method we chose to assess sample change was performed by sputter etching through the vitreous ice layer to access the hydrated bulk pHEMA film, then engaging cycles of analysis by Bi_3^{++} primary ion beam in ToF-SIMS and monochromated Al X-Rays in XPS. It should be noted that sputter etching results in surface activation as a result of the collision cascade from rastering the sample with cluster beam. Sample temperatures can be increased above -120°C to promote surface sublimation of ice, however this usually results in a dehydrated surface region²³, where precise control is needed to minimise the ice layer on the surface (0-2 nm) to allow detection of underlying material of interest¹¹⁵.

The XPS Ar-GCIB sputter depth profile to the interface between ice film and pHEMA is presented in *Figure 51a*. Etching was performed until a steady state concentration was observed with respect to C1s atomic concentration as this indicated that the bulk of the pHEMA gel had been accessed. The profile shows that the O1s concentration decreases and C1s increases to a steady state at around 2000 seconds of etching, at which point the sample area was repeatedly analysed in a cycle of 3 minute analysis and 12 minutes pause between analysis.

The results of this are shown in *Figure 51a-d*. The changes in atomic concentrations in *Figure 51b* indicate increasing amount of oxygen is present in the top 10nm of the sample surface with increasing analysis time, whilst less carbon and nitrogen is present. The ratio plots in *Figure 51c-d* better illustrate this increase. An increase in the O/C and O/N ratio is observed which appears to be relatively linear suggesting a steady increase of oxygen deposition at the surface.

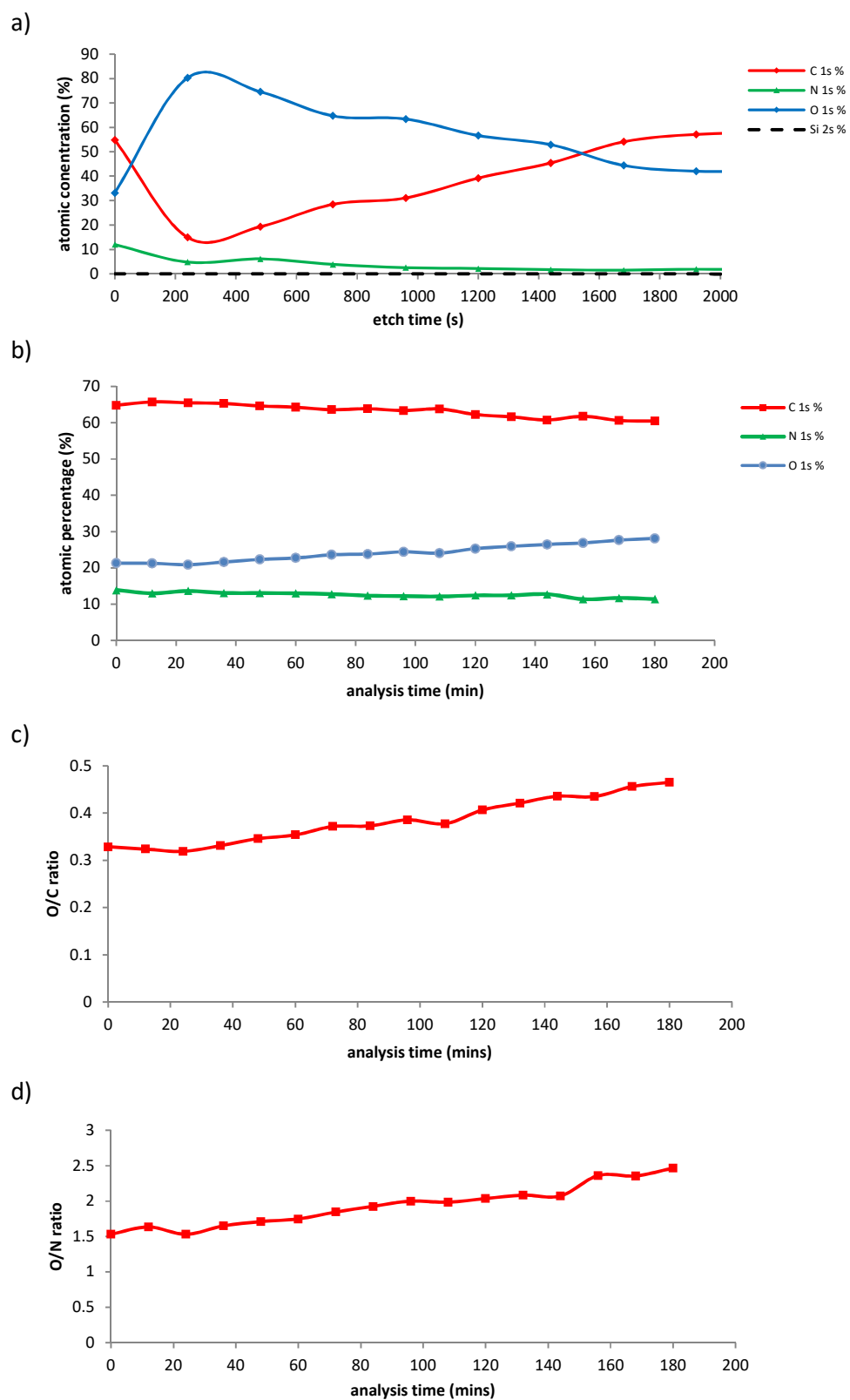
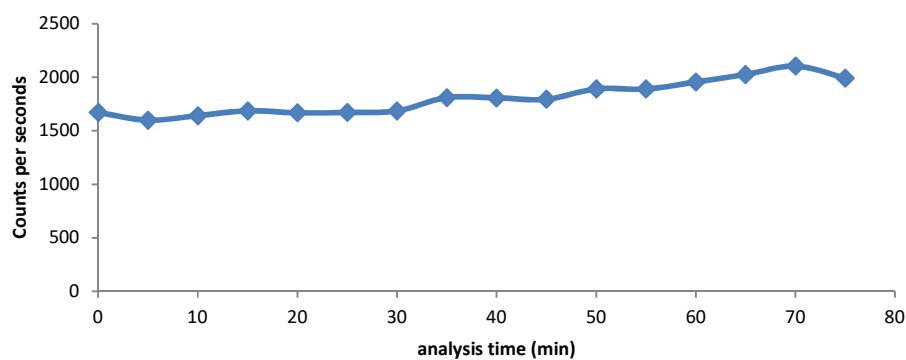


Figure 51 - xps analysis of of pHEMA hydrogels incubated in water/lysozyme for 16 hours at 20°C.

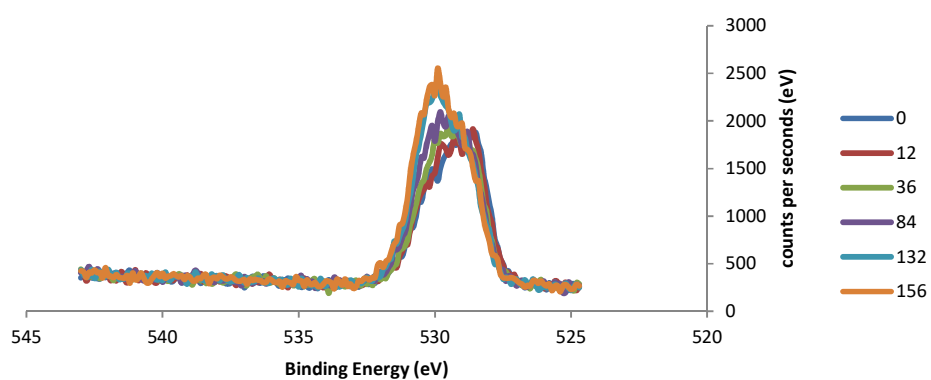
a) Ar-GCIB sputter depth profiling to interface between pHEMA film and ice. b) repeated analysis of sputtered crater over 180 minutes. c) O/C ratio change and d) O/N ratio change

High-resolution spectra in the binding energy of 543 to 524 eV, containing the uncorrected O1s envelope were recorded at each interval, shown in **Figure 52**. There is a significant change in the shape of the O1s envelope over the time period of the experiment. The peak shape shifts to the left and the measured counts increase from 0 to 156 minutes. A depiction of the fitting constraints is shown in **Figure 52**. The peak shifting occurs as a higher contribution of hydroxide O-H bonding is observed as compared to the C-O bonding contribution from the pHEMA.

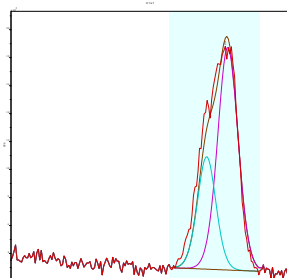
A)



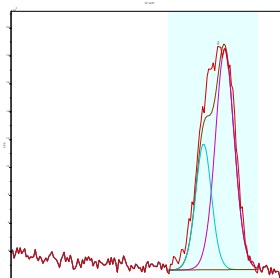
b)



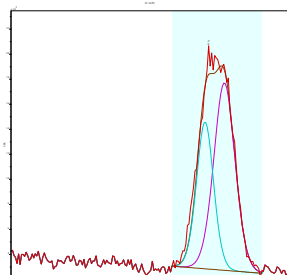
c) 0



d) 12



e) 84



f) 120

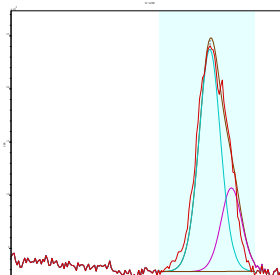


Figure 52 - Repeated x-ray analysis of sputtered crater over 180 minutes.

a) Measuring intensity change in O1s region over 180 minutes. b) Changes in the O1s envelope over 180 minutes, c-f) peak showing component fit for 0, 15, 35 and 55 minute analysis points.

High resolution peak fitting of the C1s peak in the binding energy region of 310 to 270 eV shows that the peak constraints suitable to identify the 3 specific bonding environments mentioned. Analysis of each of the percentage contributions (*Figure 53*) of the C-C, O-C-O and O-C=O in the C1s envelope over time allows us to determine if the native pHEMA is being degraded, evidenced by a change in bond contribution. The bond percentage contribution in *Figure 53* remains constant for each of the peak fitted components, indicating no damage is occurring to the pHEMA.

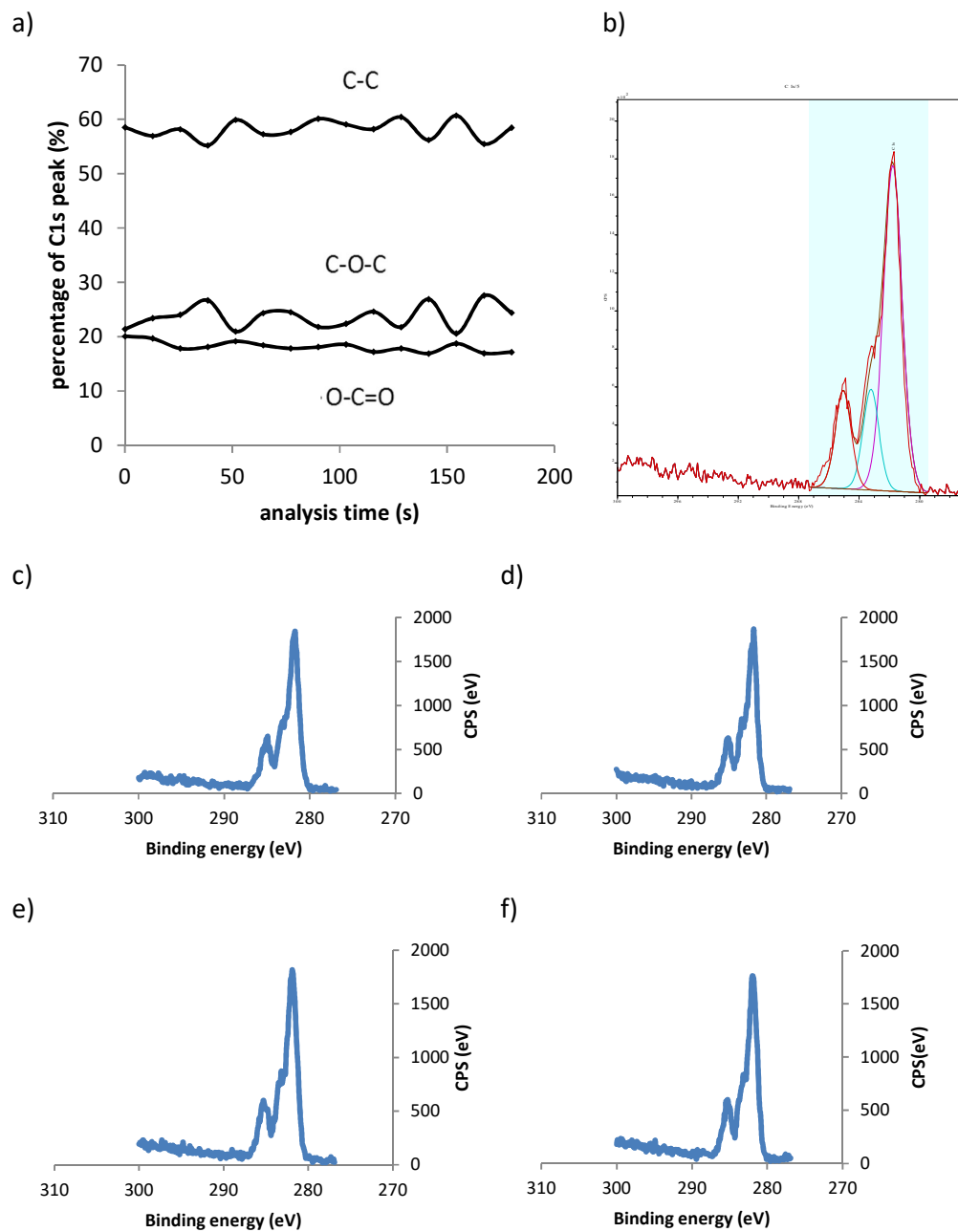


Figure 53 - Repeated x-ray analysis of sputtered crater over 180 minutes.

a) Bond contribution percentage change in c1s peak over 180 minutes. b) Changes in the c1s envelope after 180 minutes, c-f) C1s peak for 0, 15, 35 and 55 minute analysis points.

The corresponding analysis of the pHEMA hydrogel through ToF-SIMS is shown **Figure 54**. Depth profiling to the interface between hydrogel and ice similarly to XPS was performed. The interface and point where repeated analysis was performed was determined when a steady state concentration of $C_6H_9O_2^+$ was achieved signifying the bulk of the pHEMA film had been accessed. Lateral distribution of pHEMA and Ice on the surface prior to etching was determined which is shown in **Figure 55**. These images show that the distribution of ice and pHEMA is relatively homogeneous across the 100 x 100 micron area in the surface. After engaging the analysis mode for repeated static analysis of the same area monitoring of the changes in sample composition was performed determining the changes in ion intensity of ice ($H_{13}O_6^+$), pHEMA ($C_6H_9O_2^+$) and lysozyme ($C_4H_6N_2^+$). Similarly to XPS an increase in water/ice at surface is observed evidenced by an increase in the ion intensity of $H_{13}O_6^+$ and a decrease in $C_6H_9O_2^+$. The lateral distribution ion images show that features are present that are complementary with respect to $C_6H_9O_2^+$ and $H_{13}O_6^+$ (**Figure 55**). In the analysis area (100 x 100 μm) of the surface of the etch crater (500 x 500 μm), ice ($H_6O_{13}^+$) appears to be uniformly distributed and the same for pHEMA ($C_6H_9O_2^+$). After 24 minutes analysis features develop in the $H_{13}O_6^+$ ion distribution images, which are complementary to the $C_6H_9O_2^+$ distribution, which suggests water is condensing on the sample then freezing covering the pHEMA.

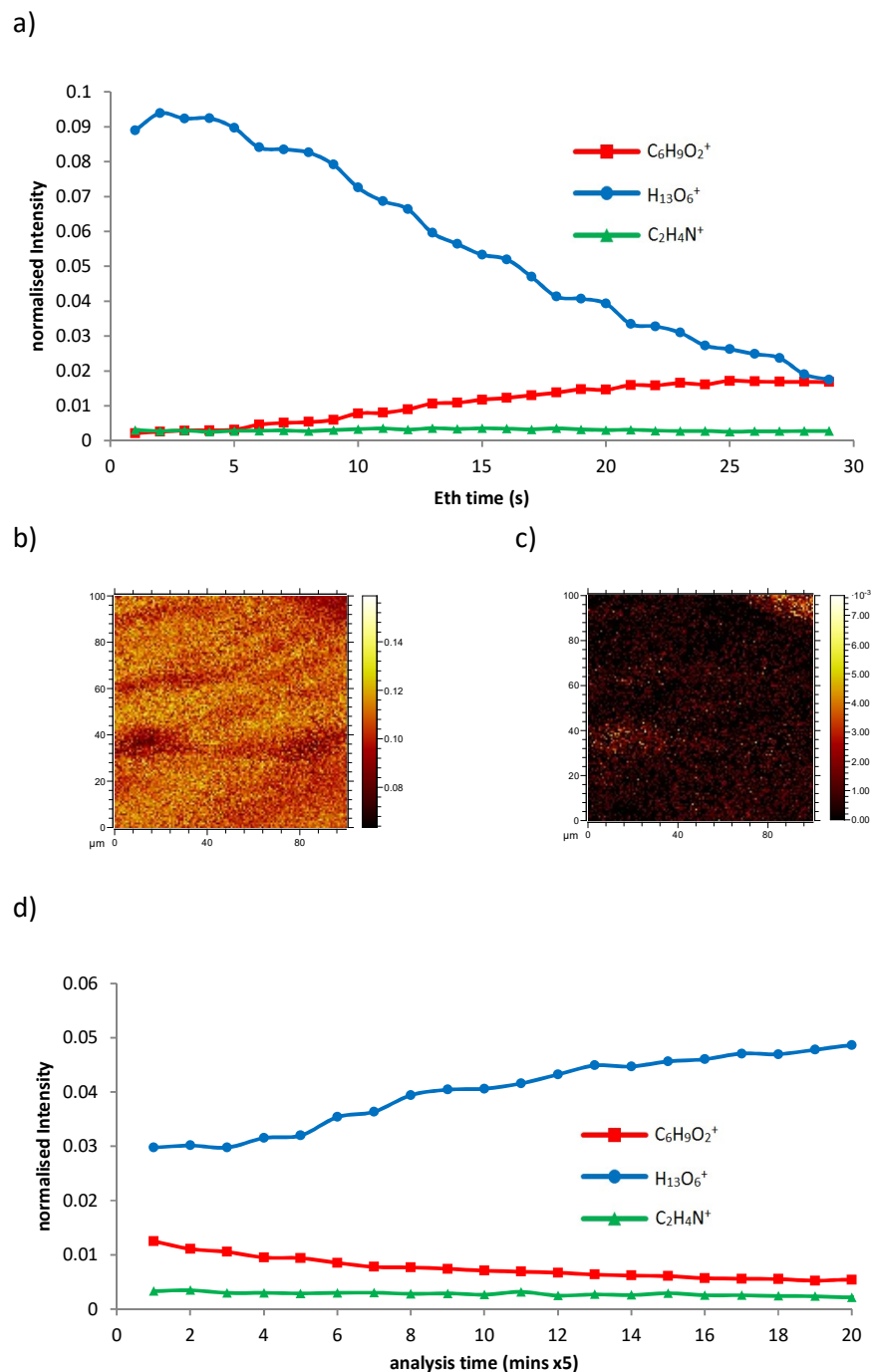


Figure 54 - Positive polarity ToF-SIMS spectra acquired from repeated scans of the hydrogel etch crater.

$C_4H_6N_2^+$ representing lysozyme is shown in green, $H_{13}O_6^+$ signifying water is represented in blue, whilst pHEMA is represented in red by $C_6H_9O_2^+$. A) Ar-sputter etching to interface between pHEMA film and vitreous ice layer on the surface. B) Lateral distribution of ice and C) pHEMA on the surface pre etch normalised to total counts. d) Damage occurring on activated surfaces, repeated analysis of the etch crater over 100 minutes.

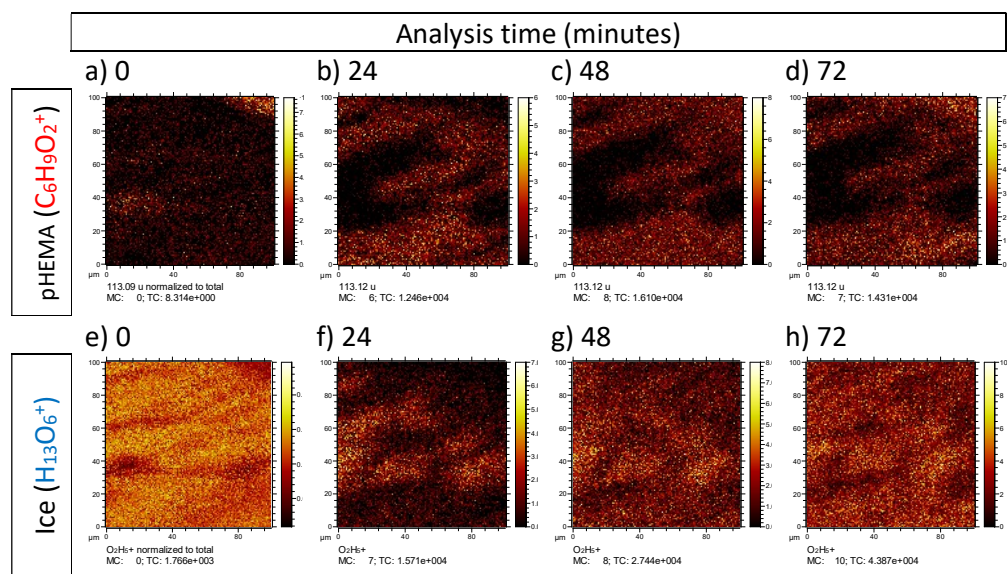


Figure 55 - Lateral distribution of pHEMA (a-d) and Ice (e-h) over 0-30 minutes repeated analysis.

6.4. DISCUSSION

Sublimation of ice from may be influenced by a number of factors. Rastering of the sample surface using a high spatially resolved analysis beam in ToF-SIMS results in a build-up of positive charge. Charge build up is neutralised by the use of charge neutralising source by either a low energy electron filament or flood gun in ToF-SIMS or XPS respectively. Analysis and charge neutralisation results in transfer of energy to the surface region of the sample which may catalyse a rise in sample temperature increasing the rate of ice sublimation in the surface region.

The increased oxygen atomic concentration at the pHEMA etch crater surface in *Figure 52a* suggests that oxygen containing species are condensing on the sample surface which is increasing the ratio of oxygen to carbon as the oxygen containing species cover the pHEMA hydrogel. As the sample is frozen hydrated, and the sample stub has been snap frozen, it is reasonable to suggest that water from the sample or surrounding sample mount has condensed on the sample under these measurement conditions.

The high resolution scans in *Figure 52b* give more information on the specific bond contributions in the O1s envelope, allowing the assessment of contribution from H-O-H bonding from water and C-O bonding from organic components. Interpretation of O1s spectra is not straightforward as apart from metal oxides, the O1s binding energy of many compounds and species falls within a very narrow range (525 to 535 eV), and the peaks tend to be broad with multiple overlapping components.

In the case of pHEMA, the pHEMA methacryl C-O bonding will contribute to the overall peak intensity and shape, along with H-O-H bonding contributed by water within the hydrogel. We observe that a higher contribution from H-O-H bonding occurs as a result of sample analysis time indicating contribution from hydroxyl bonding associated with water.

This may be either an effector of sample warming through analysis which results in the temperature of the sample increasing above the point where vacuum induced sublimation of ice occurs or the sample temperature is not low enough to prevent ice sublimation in this sample type. The impact of increased sublimation of ice on analysing hydrogels by ToF-SIMS/XPS in a frozen hydrated format, may be that the sample becomes increasingly freeze-dried. This may alter the physical structure of the hydrogel film i.e. shrinkage.

Determining contribution of specific bonds to the oxygen 1s peak is complex when compared to fitting of C1s peaks, as this is rather easier. The C1s peak for polymers usually contains three easily distinguishable components, C-C, C-O-C and O-C=O bonding. The percentage contribution of each bond to the overall C1s atomic concentration is unchanged through the experiment indicating that no change in the bond contribution is occurring in pHEMA (**Figure 53**) which would signify change in chemical state or damage.

The comparative damage study performed by analysis of the pHEMA hydrogel films by ToF-SIMS was performed similarly by reanalysing the etched area at hydrogel/ice film interface. Reanalysing the activated etch crater surface allowed us to assess the sample response in terms of chemical change under static

conditions. The primary dose of 6.84×10^{12} ions/cm² for the Bi₃⁺⁺ analysis beam was below the static limit, thus allowing reanalysis of the 100 x 100 µm area whilst preventing subsurface damage. This usually manifests as loss in molecular signal or relative peak intensities until the signal vanishes completely as the whole area is damaged. This was not observed reanalysing the same area, therefore the calculated ion dose was suitable to limit damage to the top pHEMA surface analysed.

An O/C ratio of 0.5 equates to a dehydrated pHEMA film, the ratio at the interface at the point where static reanalysis conditions were employed was measured at 1:1 H₁₃O₆⁺/C₆H₉O₂⁺. Previous depth profiling of these pHEMA hydrogel thicknesses determined that a decreasing H₁₃O₆⁺ peak intensity was observed with increasing etching depth at steady state C₆H₉O₂⁺ ion intensity. Stopping etching at the C₆H₉O₂⁺ steady state allows us to assess the sample changes in response to the experimental environment in the bulk pHEMA film, rather than assessing change in the interface regions. The presence of features in the lateral ion distribution of H₁₃O₆⁺ indicate differences of height on the pHEMA surface, whereas the observable pHEMA mass peak at 113.086 indicates that the vitrified ice layer on the surface has localised areas of where the pHEMA film is exposed.

The complementary distribution of C₆H₉O₂⁺ and H₁₃O₂⁺ after 24 minutes analysis is absent in the ion distribution images at 72 minutes. The increased ion signal of H₁₃O₂⁺ indicated that more ice is present at the surface, the longer the sample is exposed to analysis. This demonstrates that water is condensing on the hydrogel

surface, then freezing resulting in the loss of complementary ion distribution of $\text{H}_{13}\text{O}_6^+$ and $\text{C}_6\text{H}_9\text{O}_2^+$.

The increasing $\text{H}_{13}\text{O}_6^+$ peak intensity suggesting water deposition as a function of reanalysis is similarly seen in the XPS study by increase of the O1s envelope. This suggests the same effect in sample analysis is occurring in both UHV techniques. The experimental conditions employed results in sublimation of ice in the sample or surrounding sample, which condenses on the surface and freezes to form ice. In a 3D study the pause time for analysis does not allow this mass transfer effect to occur, and any resultant frosting will be removed through the etching process. Unfortunately etching results in increasing amounts of ice sublimation in the hydrogel film, preventing a steady state $\text{H}_{13}\text{O}_6^+$ ion intensity from being achieved.

6.5. CONCLUSION

It has been demonstrated that frozen-hydrated pHEMA hydrogels do not remain inert under static conditions under ToF-SIMS and XPS. Increasing deposition of ice on the sample surface occurs the further the hydrogel surface is analysed.

THESIS CONCLUSION

A sample preparation procedure using argon gas in an “air gun” style to remove the surface frost accumulated on the cooled sample surface on exposure to ambient atmosphere has been developed for analysis of frozen-hydrated hydrogels by ToF-SIMS. The methodology allows full through-thickness chemical characterisation of pHEMA hydrogel by ToF-SIMS.

Determining lysozyme distribution in pHEMA hydrogels in a frozen-hydrated state by ToF-SIMS is now achievable, where we found that the mode of lysozyme introduction affects the mobility of lysozyme within the pHEMA hydrogel. When lysozyme is combined with pHEMA into the dip coating solution, it diffuses out of the hydrogel resulting in an increased loading at the surface.

A modified preparatory procedure was also suitable for introduction of frozen-hydrated pHEMA/lysozyme hydrogels into XPS instruments. The intensities of markers attributed to lysozyme in ToF-SIMS, show a linear change as compared to the N/C ratios acquired through XPS, meaning that quantitative determination of lysozyme distribution from ToF-SIMS data alone is possible.

We have shown that condensation of ice occurs on the sample surface with increasing deposition occurring with increasing cycles of analysis under static conditions, whereas depth profiling hydrogel films of 4.2 μm thicknesses become increasingly dehydrated as a result of analysis condition in both ToF-SIMS and XPS.

This is important for gel analysis as analysing the surface of pHEMA films through ToF-SIMS and XPS will result in a steadily decrease in the ability to determine the

surface chemistry of the hydrogel, whilst depth profiling films of this type catalyses dehydration under vacuum which may change the physical structure of the hydrogel, redistributing components such as biomolecules within the film.

FUTURE WORK

The capacity of hydrogels to swell and maintain a certain volume of water within their physical structure is strongly dependent of the chemistry of the hydrogel, including but not limited; chemical groups and degree and type of cross-linking. The studies undertaken in this work have involved depth profiling physically cross-linked pHEMA films, which have shown to increasingly dehydrate when depth profiled. Thin pHEMA films bound by this manner dehydrate rapidly under ambient conditions, however cross-linked gels such as gelatin or peg-peptide conjugate gels are able to maintain their hydration characteristics significantly longer than pHEMA in ambient conditions. Investigation of chemically cross-linked gels may be of interest as dehydration under vacuum may differ, increasing the ability of the analyst to characterise the through-thickness gel chemistry in a frozen-hydrated state. The aim of this work was to establish a procedure for determining biomolecule distributions through gels in a frozen-hydrated standard format. The challenge is now scaling up for high-throughput analysis. In Nottingham the correlation of micro-array surface properties to cell response has been successful in identifying of a new polymer class with wide biomedical applicability. Microarray characterisation has been performed in a dehydrated format. However the chemical group presentation of the microarray surface may differ in a hydrated format, as we know that certain polymers reorientate their surface chemical groups upon swelling (see chapter 1). Surface and bulk chemical analysis of this microarray format will unleash the power of combinatorial hydrogel microarrays to evolve array generations to new materials.

APPENDIX

Sample no.	Silicon wafer (mg)			Silicon/pHEMA coat (mg)			pHEMA coating (mg)			Silicon/pHEMA post incubation (mg)			pHEMA post incubation (mg)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	30.14	30.54	30.54	31.12	30.9	31.22	0.98	0.36	0.1	31.99	31.02	30.68	1.85	0.48	0.14
2	30.23	30.23	30.23	30.51	30.91	31.21	0.27	0.67	0.7	30.92	31.09	31.34	0.68	0.85	1.10
3	30.05	30.05	30.05	30.22	31.12	31.18	0.17	1.07	0.96	30.48	31.53	31.4	0.43	1.48	1.35
4	29.98	29.98	29.98	30.12	31.54	31.11	0.14	1.56	0.99	30.32	32.23	31.39	0.34	2.25	1.41
5	30.01	30.01	30.01	30.27	30.86	31.49	0.25	0.84	1.22	30.65	31.28	31.62	0.63	1.26	1.60
6	29.90	29.90	29.90	30.18	30.92	31.32	0.27	1.01	1.14	30.59	31.45	31.52	0.67	1.54	1.61
7	30.41	30.41	30.41	30.48	31.43	31.31	0.07	1.02	0.83	30.58	31.86	31.53	0.17	1.45	1.12
8	30.07	30.27	30.27	30.32	30.83	31.27	0.24	0.55	0.95	30.39	31.21	31.58	0.31	0.93	1.30
9	30.31	30.31	30.31	30.48	30.92	31.19	0.17	0.61	0.71	30.72	31.32	31.22	0.41	1.01	0.91
10	30.01	30.01	30.01	30.12	30.88	31.12	0.108	0.86	1.08	30.28	31.38	31.42	0.26	1.36	1.40
Average	30.11	30.17	30.17	30.38	31.03	31.24	0.21	0.87	1.01	30.69	31.43	31.37	0.41	0.97	1.12
SD	0.15	0.17	0.20	0.06	0.12	0.10	0.2	0.18	0.09	0.123	0.18	0.08	0.12	0.09	0.12

Supplementary figure 1 - Table of gravimetric experiment on pHEMA film swelling

	Si+	C_6H_9O_2+	O_6H_13+	Log(Si+)	Log(C_6H_9O_2+)	Log(O_6H_13+)
	27.9728	113.0921	91.688	27.9728	113.0921	91.688
Sputter Time (s)	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
3.2768	8.01	3693.6	350.3	0.9	3.5	2.5
9.8304	13.01	3368.5	365.2	1.1	3.5	2.5
16.384	4.01	2932.9	350.2	0.6	3.4	2.5
22.937	11.1	3082.3	348.0	1.0	3.4	2.5
29.491	7.1	2946.0	325.8	0.8	3.4	2.5
36.044	13.1	2727.0	378.2	1.1	3.4	2.5
42.598	41.1	2319.1	300.6	1.6	3.3	2.4
49.152	251.6	1268.2	203.7	2.4	3.1	2.3
55.705	1008.5	443.1	72.1	3.0	2.6	1.8
62.259	2344.8	118.2	31.0	3.3	2.0	1.4
68.812	4031.7	52.0	28.0	3.6	1.7	1.4
75.366	5406.7	19.0	7.0	3.7	1.2	0.8
81.921	6397.1	19.0	9.0	3.8	1.2	0.9
88.473	7621.1	12.0	9.0	3.8	1.0	0.9
95.027	8190.4	11.0	8.0	3.9	1.0	0.9
101.58	8702.5	7.0	11.0	3.9	0.8	1.0
108.13	8978.1	4.0	14.0	3.9	0.6	1.1
114.68	9193.4	9.0	2.0	3.9	0.9	0.3
121.24	9511.3	5.0	5.0	3.9	0.6	0.6
127.79	9798.5	9.0	4.0	3.9	0.9	0.6
134.34	10201.9	9.0	6.0	4.0	0.9	0.7
140.90	10156.1	6.0	6.0	4.0	0.7	0.7
147.41	10288.2	6.0	11.0	4.0	0.7	1.0
154.00	10598.1	5.0	5.0	4.0	0.6	0.6
160.56	10844.7	4.0	4.0	4.0	0.6	0.6
167.11	10652.1	4.0	7.0	4.0	0.6	0.8
173.67	10600.1	2.0	4.0	4.0	0.3	0.6
180.22	10863.2	5.0	6.0	4.0	0.6	0.7
186.77	10789.2	6.0	4.0	4.0	0.7	0.6
193.33	10586.8	4.0	8.0	4.0	0.6	0.9

Supplementary figure 2 – depth profile of phema film produced by 1 dip in 1 wt/% phema

	Si+	C_6H_9O_2+	O_6H_13+	Log(Si+)	Log(C_6H_9O_2+)	Log(O_6H_13+)
	27.9728	113.0921	91.688	27.9728	113.0921	91.688
Sputter Time (s)	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
3.2768	12.0176	2169.25	279.638	3.2768	2.724204	3.33631
9.8304	8.00828	2558.02	307.932	9.8304	2.769701	3.407904
16.384	6.00611	2759.12	347.255	16.384	2.825629	3.440771
22.9376	3.00355	2771.08	350.249	22.9376	2.823044	3.442649
29.4912	8.0084	2743.68	342.321	29.4912	2.862761	3.438333
36.0448	2.00168	2677.13	346.359	36.0448	2.85969	3.427669
42.5984	6.006	2626.47	388.718	42.5984	2.869325	3.419372
49.152	16.0185	2520.56	359.521	49.152	2.904236	3.401497
55.7056	43.0466	2290.31	319.976	55.7056	2.873514	3.359894
62.2592	278.858	1280.14	209.936	62.2592	2.758353	3.107257
68.8128	1085.72	533.196	89.1943	68.8128	2.386845	2.726887
75.3664	2287.93	143.283	41.0425	75.3664	2.046148	2.156195
81.92	3240.11	31.0259	12.0077	81.92	1.362235	1.491724
88.4736	3870.08	22.0105	7.003	88.4736	1.114464	1.34263
95.0272	3715.98	11.0033	3.00085	95.0272	0.90364	1.041523
101.5808	3405.64	10.0034	8.00191	101.5808	0.30162	1.000148
108.1344	2860.55	7.00256	5.00149	108.1344	0.477671	0.845257
114.688	2374.43	3.00095	3.00074	114.688	0.47768	0.477259
121.2416	1919.6	8.00314	6.00165	121.2416	0.000534	0.90326
127.7952	1575.35	3.00079	3.00059	127.7952	0.301419	0.477236
134.3488	1283.86	1.00028	2.00043	134.3488	0.000408	0.000122
140.9024	1085.21	3.0006	2.00024	140.9024	#NUM!	0.477208
147.456	908.46	5.00149	3.00054	147.456	0.477443	0.699099
154.0096	735.197	2.0004	1.00011	154.0096	0.301269	0.301117
160.5632	633.202	3.00057	0	160.5632	0.301262	0.477204

167.1168	622.094	7.00166	2.00037	167.1168	0.301321	0.845201
173.6704	548.363	1.00014	4.00032	173.6704	0.000256	6.08E-05
180.224	502.002	3.00053	1.00014	180.224	0.000191	0.477198
186.7776	422.429	1.00019	0	186.7776	#NUM!	8.25E-05
193.3312	427.443	2.0004	3.00027	193.3312	0.000182	0.301117
199.8848	395.237	1.00018	3.00043	199.8848	0.301208	7.82E-05
206.4384	357.025	0	2.00037	206.4384	0.301202	#NUM!
212.992	359.031	0	1.00008	212.992	0.301182	#NUM!
219.5456	352.987	0	1.00011	219.5456	#NUM!	#NUM!
226.0992	337.926	0	2.00018	226.0992	0.301104	#NUM!
232.6528	338.906	3.00086	0	232.6528	0.000122	0.477246
239.2064	360.009	0	1.00007	239.2064	#NUM!	#NUM!

Supplementary figure 3 - depth profile of phema film produced by 1 dip in 1 wt/% phema

	Si+	C_6H_9O_2+	O_6H_13+	Log(Si+)	Log(C_6H_9O_2+)	Log(O_6H_13+)
	27.9728	113.0921	91.688	27.9728	113.0921	91.688
Sputter Time (s)	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
3.2768	6.00739	3084.92	317.214	0.778686	3.489244	2.501352
9.8304	6.00599	2967.15	341.423	0.778585	3.47234	2.533293
16.384	6.00487	3028.38	351.482	0.778504	3.48121	2.545903
22.9376	7.00661	2947.96	377.811	0.845508	3.469522	2.577275
29.4912	5.00448	2999	399.837	0.699359	3.476976	2.601883
36.0448	8.00716	2708.69	357.402	0.903479	3.432759	2.553157
42.5984	2.00186	2757.58	344.472	0.301434	3.440528	2.537154
49.152	5.00379	2582.92	344.407	0.699299	3.412111	2.537072
55.7056	12.0135	2532.61	337.302	1.07967	3.403568	2.528019
62.2592	6.00684	2583.94	336.367	0.778646	3.412282	2.526813
68.8128	13.0145	2579.91	349.52	1.114427	3.411605	2.543472
75.3664	11.0078	2427.6	354.455	1.041701	3.385177	2.549561
81.92	21.0235	2187.42	336.059	1.322705	3.339932	2.526416
88.4736	62.0763	1579.06	234.159	1.792926	3.198399	2.369511
95.0272	385.383	822.109	164.565	2.585893	2.914929	2.216337
101.5808	1073.55	280.997	71.1187	3.030822	2.448702	1.851984
108.1344	1988.42	96.1499	27.0224	3.298508	1.982949	1.431724
114.688	3079.72	39.0376	15.0085	3.488511	1.591483	1.176337
121.2416	3865.51	16.0101	15.0105	3.587207	1.204394	1.176395
127.7952	4457.78	10.006	11.005	3.649119	1.00026	1.04159
134.3488	4661.49	10.005	9.00527	3.668525	1.000217	0.954497
140.9024	4685.25	10.0047	12.0069	3.670733	1.000204	1.079431
147.456	4451.74	6.00237	8.00371	3.64853	0.778323	0.903291
154.0096	4091.2	9.00436	9.00395	3.611851	0.954453	0.954433
160.5632	3780.6	8.00298	5.00221	3.577561	0.903252	0.699162
167.1168	3575.22	6.00205	4.00198	3.553303	0.7783	0.602275
173.6704	3107.01	5.00195	8.00348	3.492343	0.699139	0.903279
180.224	2775.05	5.00175	9.00251	3.443271	0.699122	0.954364
186.7776	2413.97	9.00372	5.00169	3.382732	0.954422	0.699117
193.3312	1998.84	6.00344	3.00117	3.300778	0.7784	0.477291

Supplementary figure 4 - depth profile of phema film produced by 1 dip in 1 wt/% phema

Sample	Silicon wafer mass (g)			Non-Swelled pHEMA mass (g)			Hydrated pHEMA mass (g)		
	1	2	3	1	2	3	1	2	3
1	30.46	29.14	30.47	30.12	29.47	30.72	30.22	29.26	30.30
2	30.44	30.43	30.37	30.51	30.63	30.41	30.58	30.68	30.33
3	30.15	29.86	30.65	30.22	30.31	30.68	330.28	30.35	30.92
4	29.98	30.01	330.23	30.12	30.21	30.21	30.18	30.79	30.19
5	30.336	31.21	30.40	30.40	30.46	30.79	30.27	30.28	30.62
6	30.23	30.86	30.48	30.18	30.82	30.52	30.32	30.92	30.52
7	30.42	30.13	30.41	30.48	30.35	30.41	30.31	30.86	30.23
8	30.28	30.33	30.23	30.32	30.53	30.27	30.38	30.41	30.58
9	30.31	30.71	30.87	30.48	30.92	30.19	30.32	30.82	30.52
10	30.02	29.89	29.31	30.12	30.08	30.02	30.18	29.98	30.42
Average	30.265	30.257	30.342	30.295	30.378	30.422	30.304	30.435	30.463
SD	0.1611	0.5594	0.3883	0.1539	0.391	0.2416	0.1104	0.4867	0.2070

Supplementary figure 5 - difference between numbers of 1% phema coatings applied to the silicon wafers in relation to mass

Sputter Time (s)	Normalised to total counts						
	total	Si	O_6H_13+	C_6H_9O_2	Si+	O_6H_13+	C_6H_9O_2
	N/A	27.9722	91.688	113.0466	27.9722	91.688	113.0466
Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
0	79829	0	3227	6	0	0.040424	7.51607E-05
2.99988	175918	1	13760	58	5.68E-06	0.078218	0.00032969
5.99976	194147	0	15666	145	0	0.080691	0.00074685
8.99964	204897	2	16364	192	9.76E-06	0.079865	0.00093705
11.9995	207077	0	15860	258	0	0.07659	0.00124591
14.9994	219287	0	16735	365	0	0.076316	0.00166448
17.9993	225236	0	16623	446	0	0.073803	0.00198014
20.9992	233513	0	16868	585	0	0.072236	0.00250521
23.999	241995	0	17262	693	0	0.071332	0.00286369
26.9989	249002	1	17743	754	4.02E-06	0.071256	0.00302808
29.9988	251766	1	17249	832	3.97E-06	0.068512	0.00330465
32.9987	258089	3	17455	920	1.16E-05	0.067632	0.00356466
35.9986	256035	1	17006	1019	3.91E-06	0.066421	0.00397992
38.9984	263982	0	17190	1061	0	0.065118	0.00401921
41.9983	255684	3	15708	1129	1.17E-05	0.061435	0.00441560
44.9982	270571	1	16972	1226	3.7E-06	0.062727	0.00453115
47.9981	276291	2	17038	1404	7.24E-06	0.061667	0.00508159
50.998	283600	0	16981	1464	0	0.059877	0.0051622
53.9978	293906	1	17724	1666	3.4E-06	0.060305	0.00566847
56.9977	298793	2	17741	1696	6.69E-06	0.059376	0.00567617
59.9976	299610	1	17836	1848	3.34E-06	0.059531	0.00616801
62.9975	297283	2	17626	1955	6.73E-06	0.05929	0.00657622
65.9974	292540	5	17593	1768	1.71E-05	0.060139	0.00604361
68.9972	292234	8	17419	1791	2.74E-05	0.059606	0.00612865
71.9971	286816	5	17153	1788	1.74E-05	0.059805	0.00623396
74.997	283753	5	16731	1794	1.76E-05	0.058963	0.0063224
77.9969	285617	8	16554	1920	2.8E-05	0.057959	0.00672228
80.9968	280585	8	16112	1869	2.85E-05	0.057423	0.00666108
83.9966	273000	13	15483	1876	4.76E-05	0.056714	0.00687179
86.9965	266180	7	14825	1922	2.63E-05	0.055695	0.00722067
89.9964	260060	15	14262	1967	5.77E-05	0.054841	0.00756363
92.9963	252107	16	13436	1979	6.35E-05	0.053295	0.00784984
95.9962	241042	8	12588	2040	3.32E-05	0.052223	0.00846325
98.996	232936	15	12111	1859	6.44E-05	0.051993	0.00798073
101.996	218707	12	11232	1829	5.49E-05	0.051356	0.00836278
104.996	210537	11	10739	1779	5.22E-05	0.051008	0.00844982
107.996	204350	16	10149	1788	7.83E-05	0.049665	0.00874969
110.996	196607	16	9653	1776	8.14E-05	0.049098	0.00903324
113.995	191182	9	9126	1771	4.71E-05	0.047735	0.00926342
116.995	182474	6	8432	1739	3.29E-05	0.046209	0.00953012
119.995	177794	14	7863	1682	7.87E-05	0.044225	0.00946038
122.995	170408	15	7148	1738	8.8E-05	0.041946	0.01019905
125.995	160540	11	6418	1723	6.85E-05	0.039978	0.01073252
128.995	153516	18	5816	1684	0.000117	0.037885	0.01096954
131.995	150592	9	5495	1800	5.98E-05	0.036489	0.01195282
134.995	144901	8	4826	1823	5.52E-05	0.033305	0.01258100
137.994	139728	8	4302	1837	5.73E-05	0.030788	0.01314697
140.994	136541	10	4032	1873	7.32E-05	0.02953	0.01371749
143.994	134181	5	3640	1853	3.73E-05	0.027128	0.01380970
146.994	126592	3	3006	1821	2.37E-05	0.023746	0.01438479
149.994	121266	5	2393	1843	4.12E-05	0.019733	0.01519799
152.994	119272	4	2019	2008	3.35E-05	0.016928	0.01683546
155.994	116773	4	1612	1996	3.43E-05	0.013805	0.01709299
158.994	114041	4	1270	2083	3.51E-05	0.011136	0.01826536
161.994	112362	3	975	2065	2.67E-05	0.008677	0.01837809
164.993	108825	3	688	1985	2.76E-05	0.006322	0.01824029
167.993	103437	7	294	1998	6.77E-05	0.002842	0.01931610
170.993	101379	2	256	2004	1.97E-05	0.002525	0.01976740
173.993	101399	1	136	2010	9.86E-06	0.001341	0.01982268
176.993	101283	3	111	2071	2.96E-05	0.001096	0.02044765
179.993	103676	3	98	2056	2.89E-05	0.000945	0.01983101
182.993	107222	2	84	2102	1.87E-05	0.000783	0.01960418

185.993	107189	4	80	2131	3.73E-05	0.000746	0.01988077
188.992	107912	6	68	2227	5.56E-05	0.00063	0.02063718
191.992	108665	9	68	2216	8.28E-05	0.000626	0.02039295
194.992	105025	10	53	2219	9.52E-05	0.000505	0.02112830
197.992	103720	7	67	2141	6.75E-05	0.000646	0.02064211
200.992	104136	8	45	2179	7.68E-05	0.000432	0.02092456
203.992	105263	10	54	2226	9.5E-05	0.000513	0.02114703
206.992	103373	6	39	2123	5.8E-05	0.000377	0.02053727
209.992	106968	5	51	2208	4.67E-05	0.000477	0.02064168
212.991	105462	9	37	2146	8.53E-05	0.000351	0.02034856
215.991	105178	8	36	2164	7.61E-05	0.000342	0.02057464
218.991	103089	10	45	2111	9.7E-05	0.000437	0.02047745
221.991	102678	8	44	2136	7.79E-05	0.000429	0.02080289
224.991	97655	9	35	1949	9.22E-05	0.000358	0.01995801
227.991	98491	6	29	2050	6.09E-05	0.000294	0.02081408
230.991	96371	11	27	1967	0.000114	0.00028	0.02041070
233.991	95079	11	29	2035	0.000116	0.000305	0.02140325
236.991	95018	7	23	1989	7.37E-05	0.000242	0.02093287
239.99	94540	2	29	1939	2.12E-05	0.000307	0.02050983
242.99	90838	13	15	1937	0.000143	0.000165	0.02132367
245.99	84709	5	22	1727	5.9E-05	0.00026	0.02038744
248.99	81597	10	27	1699	0.000123	0.000331	0.02082184
251.99	76481	14	18	1646	0.000183	0.000235	0.02152168
254.99	71172	7	16	1543	9.84E-05	0.000225	0.02167987
257.99	64652	7	20	1347	0.000108	0.000309	0.02083462
260.99	60361	11	17	1336	0.000182	0.000282	0.02213349
263.989	54591	9	6	1182	0.000165	0.00011	0.02165192
266.989	53205	6	11	1122	0.000113	0.000207	0.02108824
269.989	48917	9	13	1055	0.000184	0.000266	0.02156714
272.989	44046	5	10	948	0.000114	0.000227	0.02152295
275.989	40662	9	14	880	0.000221	0.000344	0.02164182
278.989	36018	15.9	10	835	0.000441	0.000278	0.02318285
281.989	33152	23.49	8	733	0.000709	0.000241	0.02211028
284.989	29309	31.839	7	675	0.001086	0.000239	0.02303046
287.988	27284	41.0229	10	614	0.001504	0.000367	0.02250403
290.988	24785	51.1251	5	564	0.002063	0.000202	0.02275569
293.988	24697	62.2377	7	535	0.00252	0.000283	0.02166255
296.988	21861	74.4614	6	502	0.003406	0.000274	0.02296326
299.988	19656	87.9076	2	459	0.004472	0.000102	0.02335164
302.988	18283	102.698	3	442	0.005617	0.000164	0.02417546
305.988	16972	118.968	2	363	0.00701	0.000118	0.02138816
308.988	15294	136.865	2	376	0.008949	0.000131	0.02458480
311.988	13635	156.551	0	321	0.011482	0	0.02354235
314.987	13436	178.206	3	300	0.013263	0.000223	0.02232807
317.987	11886	202.027	2	268	0.016997	0.000168	0.02254753
320.987	10331	228.230	1	256	0.022092	9.68E-05	0.02477978
323.987	8719	257.053	6	203	0.029482	0.000688	0.02328248
326.987	7323	288.758	1	174	0.039432	0.000137	0.02376075
329.987	6715	323.634	1	153	0.048196	0.000149	0.02278481
332.987	5806	361.997	2	115	0.062349	0.000344	0.01980709

Supplementary figure 6 – ToF-SIMS depth profile of pHEMA stage frozen non airgun

Sputter Time (s)	Normalised to total counts						
	total	Si+	O_6H_13+	C_6H_9O_2	Si+	O_6H_13+	C_6H_9O_2
	N/A	27.9733	91.688	113.0466	27.9733	91.688	113.0466
Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
0	9426	0	711	452	0	0.07543	0.047952
4.99986	7296	0	474	403	0	0.064967	0.055236
9.99972	7688	0	473	399	0	0.061524	0.051899
14.9996	8046	0	495	453	0	0.061521	0.056301
19.9994	9303	0	588	484	0	0.063205	0.052026
24.9993	9992	0	620	492	0	0.06205	0.049239
29.9992	10676	0	600	499	0	0.056201	0.04674
34.999	11058	0	576	526	0	0.052089	0.047567
39.9989	11041	0	554	511	0	0.050177	0.046282
44.9987	11166	0	583	536	0	0.052212	0.048003
49.9986	11145	1	573	450	8.97E-05	0.051413	0.040377
54.9985	10787	1	561	481	9.27E-05	0.052007	0.044591
59.9983	10063	0	556	448	0	0.055252	0.04452
64.9982	9007	0	511	389	0	0.056734	0.043189
69.998	8588	0	454	401	0	0.052864	0.046693
74.9979	8775	0	499	389	0	0.056866	0.04433
79.9978	8943	0	475	398	0	0.053114	0.044504
84.9976	7658	0	416	357	0	0.054322	0.046618
89.9975	7803	0	425	386	0	0.054466	0.049468
94.9973	8144	1	455	363	0.000123	0.055869	0.044573
99.9972	8348	0	496	368	0	0.059415	0.044082
104.997	8287	1	459	379	0.000121	0.055388	0.045734
109.997	8059	0	468	335	0	0.058072	0.041568
114.997	7110	0	435	326	0	0.061181	0.045851
119.997	7038	0	367	285	0	0.052145	0.040494
124.997	7020	0	387	339	0	0.055128	0.048291
129.996	6527	1	352	282	0.000153	0.05393	0.043205
134.996	6480	0	381	289	0	0.058796	0.044599
139.996	6437	0	381	265	0	0.059189	0.041168
144.996	6683	2	381	282	0.000299	0.05701	0.042197
149.996	6856	0	381	289	0	0.055572	0.042153
154.996	7065	1	416	306	0.000142	0.058882	0.043312
159.996	7289	2	449	326	0.000274	0.0616	0.044725
164.995	5899	0	333	258	0	0.05645	0.043736
169.995	5520	0	324	260	0	0.058696	0.047101
174.995	5635	1	311	238	0.000177	0.055191	0.042236
179.995	6090	1	355	231	0.000164	0.058292	0.037931
184.995	6313	1	411	233	0.000158	0.065104	0.036908
189.995	5157	1	291	214	0.000194	0.056428	0.041497
194.995	5754	1	319	245	0.000174	0.05544	0.042579
199.994	5831	2	331	249	0.000343	0.056766	0.042703
204.994	5990	2	377	245	0.000334	0.062938	0.040902
209.994	5992	3	370	258	0.000501	0.061749	0.043057
214.994	5326	3	290	212	0.000563	0.05445	0.039805
219.994	4670	2	284	185	0.000428	0.060814	0.039615
224.994	4134	4	231	163	0.000968	0.055878	0.039429
229.994	4236	7	212	164	0.001653	0.050047	0.038716
234.993	4204	8	243	153	0.001903	0.057802	0.036394
239.993	4338	17	242	139	0.003919	0.055786	0.032042
244.993	4585	36	254	174	0.007852	0.055398	0.03795
249.993	4528	39	209	169	0.008613	0.046157	0.037323
254.993	4864	57	221	147	0.011719	0.045436	0.030222
259.993	4081	72	200	118	0.017643	0.049008	0.028914
264.993	3901	107	171	120	0.027429	0.043835	0.030761
269.992	4115	142	172	104	0.034508	0.041798	0.025273
274.992	4793	178	173	143	0.037137	0.036094	0.029835
279.992	5741	215	234	164	0.03745	0.040759	0.028566
284.992	4838	309	150	133	0.063869	0.031005	0.027491
289.992	6188	371	223	156	0.059955	0.036037	0.02521
294.992	7163	402	238	189	0.056122	0.033226	0.026386
299.992	8492	511	281	253	0.060174	0.03309	0.029793
304.991	9574	579	348	303	0.060476	0.036348	0.031648

309.991	11698	679	462	380	0.058044	0.039494	0.032484
314.991	11805	716	473	378	0.060652	0.040068	0.03202
319.991	13143	845	562	465	0.064293	0.04276	0.03538
324.991	13970	1017	586	509	0.072799	0.041947	0.036435
329.991	15708	1277	647	548	0.081296	0.041189	0.034887
334.991	17132	1356	771	610	0.07915	0.045004	0.035606
339.99	18025	1536	818	644	0.085215	0.045381	0.035728
344.99	19185	1767	887	655	0.092103	0.046234	0.034141
349.99	19513	1818	869	661	0.093169	0.044534	0.033875
354.99	20557	2235	892	714	0.108722	0.043392	0.034733
359.99	19669	2238	827	698	0.113783	0.042046	0.035487
364.99	19808	2363	811	631	0.119295	0.040943	0.031856
369.99	20608	2671	868	662	0.12961	0.04212	0.032123
374.99	21300	2941	859	649	0.138075	0.040329	0.030469
379.989	20310	3003	830	593	0.147858	0.040867	0.029197
384.989	20194	3144	743	553	0.15569	0.036793	0.027384
389.989	20756	3465	671	523	0.16694	0.032328	0.025198
394.989	20774	3510	654	514	0.168961	0.031482	0.024742
399.989	20700	3684	610	463	0.177971	0.029469	0.022367
404.989	20028	3746	645	463	0.187038	0.032205	0.023118
409.989	19092	3859	580	413	0.202127	0.030379	0.021632
414.988	18687	3947	578	361	0.211216	0.030931	0.019318
419.988	18180	4024	456	330	0.221342	0.025083	0.018152
424.988	18591	4272	436	269	0.229789	0.023452	0.014469
429.988	18338	4340	381	276	0.236667	0.020777	0.015051
434.988	18354	4435	368	265	0.241637	0.02005	0.014438
439.988	18583	4453	388	239	0.239628	0.020879	0.012861
444.988	18686	4573	378	243	0.244729	0.020229	0.013004
449.987	18836	4530	396	221	0.240497	0.021024	0.011733
454.987	17075	4494	319	201	0.263192	0.018682	0.011772
459.987	16744	4439	314	201	0.26511	0.018753	0.012004
464.987	17349	4550	307	192	0.262263	0.017696	0.011067
469.987	17954	4613	322	179	0.256934	0.017935	0.00997
474.987	17370	4608	282	173	0.265285	0.016235	0.00996
479.987	16719	4592	282	168	0.274658	0.016867	0.010048
484.986	17287	4700	274	154	0.271881	0.01585	0.008908
489.986	17434	4692	298	155	0.269129	0.017093	0.008891
494.986	17680	4657	276	168	0.263405	0.015611	0.009502
499.986	17595	4699	281	148	0.267065	0.01597	0.008411
504.986	15920	4500	211	124	0.282663	0.013254	0.007789
509.986	15199	4441	199	95	0.29219	0.013093	0.00625
514.986	14845	4424	186	107	0.298013	0.012529	0.007208
519.985	15078	4461	151	106	0.295862	0.010015	0.00703
524.985	14812	4324	183	64	0.291925	0.012355	0.004321
529.985	15028	4509	167	74	0.30004	0.011113	0.004924
534.985	15428	4428	189	82	0.287011	0.01225	0.005315
539.985	15327	4397	193	75	0.286879	0.012592	0.004893
544.985	15490	4495	199	72	0.290187	0.012847	0.004648
549.985	15153	4427	162	70	0.292153	0.010691	0.00462
554.984	13428	4214	106	53	0.313822	0.007894	0.003947
559.984	13211	3993	116	52	0.302248	0.008781	0.003936
564.984	13580	4144	140	35	0.305155	0.010309	0.002577
569.984	14261	4228	131	38	0.296473	0.009186	0.002665
574.984	13666	4041	140	30	0.295697	0.010244	0.002195
579.984	12703	3991	112	35	0.314178	0.008817	0.002755
584.984	13358	4016	128	29	0.300644	0.009582	0.002171
589.983	13711	4071	119	31	0.296915	0.008679	0.002261
594.983	13753	4086	128	27	0.297099	0.009307	0.001963
599.983	13687	4046	141	14	0.295609	0.010302	0.001023
604.983	12634	3933	108	24	0.311303	0.008548	0.0019
609.983	11580	3708	82	22	0.320207	0.007081	0.0019
614.983	11865	3778	83	12	0.318416	0.006995	0.001011
619.983	11875	3611	87	18	0.304084	0.007326	0.001516
624.982	12015	3714	82	17	0.309114	0.006825	0.001415
629.982	12423	3837	88	13	0.308863	0.007084	0.001046
634.982	12651	3849	97	11	0.304245	0.007667	0.000869
639.982	12971	3913	124	14	0.301673	0.00956	0.001079

644.982	12916	3853	120	7	0.298312	0.009291	0.000542
649.982	12141	3669	101	8	0.302199	0.008319	0.000659
654.982	10857	3412	53	5	0.314267	0.004882	0.000461
659.982	11319	3507	73	13	0.309833	0.006449	0.001149
664.981	12117	3758	96	11	0.310143	0.007923	0.000908
669.981	12241	3622	115	8	0.295891	0.009395	0.000654
674.981	10688	3334	54	3	0.311939	0.005052	0.000281
679.981	11300	3470	85	6	0.30708	0.007522	0.000531
684.981	11492	3539	74	4	0.307953	0.006439	0.000348
689.981	11686	3581	91	4	0.306435	0.007787	0.000342
694.981	12133	3627	75	4	0.298937	0.006181	0.00033
699.98	12479	3659	101	3	0.293213	0.008094	0.00024
704.98	12352	3596	102	8	0.291127	0.008258	0.000648
709.98	11332	3458	71	5	0.305154	0.006265	0.000441
714.98	11171	3503	72	9	0.31358	0.006445	0.000806
719.98	11137	3550	74	6	0.318757	0.006645	0.000539
724.98	11413	3517	111	5	0.308157	0.009726	0.000438
729.98	11770	3554	96	12	0.301954	0.008156	0.00102
734.979	12171	3619	113	5	0.297346	0.009284	0.000411
739.979	12233	3653	97	9	0.298618	0.007929	0.000736
744.979	12632	3783	107	13	0.299478	0.008471	0.001029
749.979	12378	3718	88	9	0.300372	0.007109	0.000727
754.979	11008	3444	49	4	0.312863	0.004451	0.000363
759.979	11169	3495	89	3	0.31292	0.007968	0.000269
764.979	11733	3653	85	3	0.311344	0.007245	0.000256
769.978	12389	3553	98	0	0.286787	0.00791	0
774.978	10651	3272	68	2	0.307201	0.006384	0.000188
779.978	11367	3554	62	5	0.312659	0.005454	0.00044
784.978	11837	3566	96	3	0.301259	0.00811	0.000253
789.978	12227	3658	115	7	0.299174	0.009405	0.000573
794.978	12296	3684	110	5	0.29961	0.008946	0.000407
799.978	12363	3645	133	5	0.294831	0.010758	0.000404
804.977	12136	3636	99	3	0.299604	0.008158	0.000247
809.977	10814	3390	70	4	0.313483	0.006473	0.00037
814.977	10598	3432	46	2	0.323835	0.00434	0.000189
819.977	10844	3377	69	1	0.311416	0.006363	9.22E-05
824.977	11164	3468	68	0	0.310641	0.006091	0
829.977	11375	3522	81	3	0.309626	0.007121	0.000264
834.977	11568	3567	85	3	0.308351	0.007348	0.000259
839.976	11924	3621	94	3	0.303673	0.007883	0.000252
844.976	12189	3573	106	3	0.293133	0.008696	0.000246
849.976	12096	3554	103	4	0.293816	0.008515	0.000331
854.976	10622	3407	56	1	0.320749	0.005272	9.41E-05
859.976	11310	3501	100	2	0.309549	0.008842	0.000177
864.976	11984	3619	110	3	0.301986	0.009179	0.00025
869.976	12557	3790	134	5	0.301824	0.010671	0.000398
874.976	10790	3441	77	3	0.318906	0.007136	0.000278
879.975	11528	3588	77	10	0.311242	0.006679	0.000867
884.975	12145	3696	100	1	0.304323	0.008234	8.23E-05
889.975	12429	3714	100	4	0.298817	0.008046	0.000322
894.975	12642	3742	124	6	0.295997	0.009809	0.000475
899.975	12801	3818	124	6	0.298258	0.009687	0.000469
904.975	11755	3557	83	0	0.302595	0.007061	0
909.975	11157	3533	64	1	0.316662	0.005736	8.96E-05
914.974	11099	3511	76	2	0.316335	0.006847	0.00018
919.974	11327	3464	67	0	0.305818	0.005915	0
924.974	11565	3651	64	3	0.315694	0.005534	0.000259
929.974	11815	3630	80	2	0.307237	0.006771	0.000169
934.974	11996	3671	91	4	0.306019	0.007586	0.000333
939.974	12257	3693	93	7	0.301297	0.007588	0.000571
944.974	12717	3798	123	8	0.298655	0.009672	0.000629
949.973	11614	3618	83	2	0.311521	0.007147	0.000172
954.973	11176	3506	72	4	0.313708	0.006442	0.000358
959.973	11899	3643	102	3	0.30616	0.008572	0.000252
964.973	12625	3843	108	6	0.304396	0.008554	0.000475
969.973	11708	3656	90	3	0.312265	0.007687	0.000256
974.973	11729	3699	80	5	0.315372	0.006821	0.000426

979.973	12088	3701	82	3	0.306171	0.006784	0.000248
984.972	12381	3850	98	7	0.31096	0.007915	0.000565
989.972	12874	3921	121	2	0.304567	0.009399	0.000155
994.972	13267	3971	125	3	0.299314	0.009422	0.000226
999.972	13449	3967	141	5	0.294966	0.010484	0.000372
1004.97	12940	3926	113	3	0.3034	0.008733	0.000232
1009.97	12020	3816	80	6	0.317471	0.006656	0.000499

Supplementary figure 7 – ToF-SIMS depth profile of pHEMA snap frozen non-airgun

Sputter Time (s)	Normalised to total counts						
	total	Si+	O_6H_13+	C_6H_9O_2+	Si+	O_6H_13+	C_6H_9O_2+
	N/A	27.9836	91.688	113.0735	27.9836	91.688	113.0735
	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
0	533719	12	2631	6705	2.24837E-05	0.00493	0.012563
2.99988	451321	0	3215	10988	0	0.007124	0.024346
5.99976	450962	1	3232	10798	2.21748E-06	0.007167	0.023944
8.99964	443156	1	3192	10637	2.25654E-06	0.007203	0.024003
11.9995	444030	1	3318	10841	2.2521E-06	0.007472	0.024415
14.9994	445010	1	3304	11046	2.24714E-06	0.007425	0.024822
17.9993	447889	0	3456	11204	0	0.007716	0.025015
20.9992	446493	1	3444	11319	2.23968E-06	0.007713	0.025351
23.999	460404	2	3642	11289	4.34401E-06	0.00791	0.02452
26.9989	452567	0	3520	11263	0	0.007778	0.024887
29.9988	457468	0	3644	11288	0	0.007966	0.024675
32.9987	459326	0	3661	11452	0	0.00797	0.024932
35.9986	461168	1	3688	11545	2.16841E-06	0.007997	0.025034
38.9984	461150	2	3664	11345	4.33698E-06	0.007945	0.024602
41.9983	456352	2	3585	11486	4.38258E-06	0.007856	0.025169
44.9982	464162	0	3743	11564	0	0.008064	0.024914
47.9981	460094	2	3654	11315	4.34694E-06	0.007942	0.024593
50.998	465244	0	3821	11559	0	0.008213	0.024845
53.9978	467796	0	3826	11544	0	0.008179	0.024677
56.9977	469659	2	3733	11498	4.25841E-06	0.007948	0.024482
59.9976	473452	0	3664	11798	0	0.007739	0.024919
62.9975	470982	0	3769	11814	0	0.008002	0.025084
65.9974	482408	1	3886	11831	2.07293E-06	0.008055	0.024525
68.9972	472744	0	3834	11652	0	0.00811	0.024648
71.9971	476037	1	3726	11815	2.10068E-06	0.007827	0.024819
74.997	473855	0	3879	11544	0	0.008186	0.024362
77.9969	474829	0	3781	11467	0	0.007963	0.02415
80.9968	475179	0	3814	11789	0	0.008026	0.02481
83.9966	472127	2	3746	11745	4.23615E-06	0.007934	0.024877
86.9965	477257	1	3778	11807	2.09531E-06	0.007916	0.024739
89.9964	484590	0	3916	11687	0	0.008081	0.024117
92.9963	478681	0	3835	11733	0	0.008012	0.024511
95.9962	480378	1	3793	11732	2.08169E-06	0.007896	0.024422
98.996	476878	1	3717	11453	2.09697E-06	0.007794	0.024017
101.996	474934	1	3718	11462	2.10556E-06	0.007828	0.024134
104.996	473940	0	3592	11566	0	0.007579	0.024404
107.996	476699	0	3668	11716	0	0.007695	0.024577
110.996	478704	3	3720	11733	6.26692E-06	0.007771	0.02451
113.995	478910	0	3642	11529	0	0.007605	0.024073
116.995	471449	2	3505	11392	4.24224E-06	0.007435	0.024164
119.995	457111	2	3301	10780	4.3753E-06	0.007221	0.023583
122.995	444867	0	3154	10677	0	0.00709	0.024
125.995	422676	0	2891	10147	0	0.00684	0.024007
128.995	392550	2	2558	9267	5.09489E-06	0.006516	0.023607
131.995	294265	253	1685	6835	0.000859769	0.005726	0.023227
134.995	183424	503	800	4121	0.00274228	0.004361	0.022467
137.994	116943	617	489	2714	0.005276075	0.004182	0.023208
140.994	24869	799	83	412	0.032128353	0.003337	0.016567
143.994	7761	621	11	54	0.080015462	0.001417	0.006958

146.994	9058	551	22	83	0.060830205	0.002429	0.009163
149.994	6731	558	13	58	0.082900015	0.001931	0.008617
152.994	6087	522	9	47	0.08575653	0.001479	0.007721
155.994	5513	557	14	48	0.10103392	0.002539	0.008707
158.994	5857	491	7	53	0.083831313	0.001195	0.009049
161.994	5183	485	12	48	0.09357515	0.002315	0.009261
164.993	4724	509	5	17	0.107747671	0.001058	0.003599
167.993	4659	469	5	40	0.100665379	0.001073	0.008586
170.993	4277	469	10	29	0.109656301	0.002338	0.00678
173.993	4038	474	9	26	0.117384844	0.002229	0.006439
176.993	4092	509	6	20	0.124389052	0.001466	0.004888
179.993	4167	474	7	20	0.1137509	0.00168	0.0048
182.993	3784	464	6	22	0.122621564	0.001586	0.005814
185.993	3690	438	5	21	0.118699187	0.001355	0.005691
188.992	3651	421	2	23	0.115310874	0.000548	0.0063
191.992	3410	439	4	24	0.128739003	0.001173	0.007038
194.992	3299	454	11	22	0.13761746	0.003334	0.006669
197.992	3215	491	9	23	0.152721617	0.002799	0.007154

Supplementary figure 8 - ToF-SIMS depth profile of phema snap frozen with airgun

Sputter Time (s)	Log ion intensity					
	Si+	O_6H_13+	C_6H_9O_2+	Si+	O_6H_13+	C_6H_9O_2+
	27.9836	91.688	113.0731	27.9836	91.688	113.0731
	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
0	12.002	3549.16	7311.58	1.079254	3.550126	3.864011
2.9999	0	2077.28	12661.2	#NUM!	3.317495	4.102475
5.9998	1.00011	2195.68	12398.4	4.78E-05	3.341569	4.093366
8.9996	1.00007	2135.68	12194.1	3.04E-05	3.329536	4.08615
11.9995	1.00011	2094.26	12475.8	4.78E-05	3.321031	4.096068
14.9994	1.00009	2037.76	12733.8	3.91E-05	3.309153	4.104958
17.9993	0	2123.92	12999.4	#NUM!	3.327138	4.113923
20.9992	1.00007	2145.92	13134.7	3.04E-05	3.331614	4.11842
23.999	3.00021	2155.17	13132.4	0.477152	3.333482	4.118344
26.9989	1.00009	2126.3	13079	3.91E-05	3.327625	4.116575
29.9988	0	2197.37	13109.7	#NUM!	3.341903	4.117593
32.9987	0	2120.11	13380.8	#NUM!	3.326358	4.126482
35.9986	1.00007	2178.4	13514.1	3.04E-05	3.338138	4.130787
38.9984	2.00024	2189.19	13236.1	0.301082	3.340283	4.12176
41.9983	2.00024	2149.54	13405.7	0.301082	3.332346	4.127289
44.9982	0	2133.89	13551	#NUM!	3.329172	4.131971
47.9981	2.00022	2137.03	13202.8	0.301078	3.329811	4.120666
50.998	0	2119.12	13533.7	#NUM!	3.326156	4.131417
53.9978	0	2119.7	13499.9	#NUM!	3.326274	4.130331
56.9977	3.00015	2268.69	13488.2	0.477143	3.355775	4.129954
59.9976	0	2223	13888.1	#NUM!	3.346939	4.142643
62.9975	0	2140.6	13862.5	#NUM!	3.330536	4.141842
65.9974	1.00007	2190.16	13938.2	3.04E-05	3.340476	4.144207
68.9972	0	2250.84	13639.8	#NUM!	3.352345	4.134808
71.9971	2.00024	2141.56	13867.7	0.301082	3.33073	4.142004
74.997	0	2114.47	13521.1	#NUM!	3.325202	4.131012
77.9969	0	2121.85	13391.5	#NUM!	3.326715	4.126829
80.9968	0	2158.39	13847.9	#NUM!	3.33413	4.141384
83.9966	2.0002	2264.83	13770.7	0.301073	3.355036	4.138956
86.9965	1.00009	2205.94	13872.2	3.91E-05	3.343594	4.142145
89.9964	2.00028	2220.55	13693.4	0.301091	3.346461	4.136511
92.9963	1.00007	2165.73	13774.4	3.04E-05	3.335604	4.139073
95.9962	1.00007	2225.38	13819	3.04E-05	3.347404	4.140477
98.996	1.00005	2106.98	13399.3	2.17E-05	3.32366	4.127082
101.9959	1.00009	2223.32	13393.1	3.91E-05	3.347002	4.126881
104.9958	0	2201.09	13572.9	#NUM!	3.342638	4.132673
107.9957	0	2203.25	13682.6	#NUM!	3.343064	4.136169
110.9956	3.00025	2263.43	13766.5	0.477157	3.354767	4.138824
113.9954	1.00005	2275.98	13470.7	2.17E-05	3.357168	4.12939

116.9953	2.0002	2226.05	13239.7	0.301073	3.347535	4.121878
119.9952	2.00016	2169.64	12384.7	0.301065	3.336388	4.092885
122.9951	0	2100.61	12220	#NUM!	3.322345	4.087071
125.995	1.00007	1999.27	11484.8	3.04E-05	3.300871	4.060123
128.9948	2.0002	1775.65	10313.2	0.301073	3.249357	4.013393
131.9947	270.805	1262.54	7312.82	2.432657	3.101245	3.864085
134.9946	527.953	670.974	4228.21	2.722595	2.826706	3.626157
137.9945	643.44	364.888	2722.65	2.808508	2.56216	3.434992
140.9944	824.34	57.1145	406.141	2.916106	1.756746	2.608677
143.9942	631.189	12.0037	52.0444	2.800159	1.079315	1.716374
146.9941	567.4	13.007	82.1002	2.753889	1.114177	1.914344
149.994	575.613	9.00357	55.0463	2.760131	0.954415	1.740728
152.9939	538.153	5.00066	46.0356	2.730906	0.699027	1.663094
155.9938	564.388	12.0037	47.0359	2.751578	1.079315	1.672429
158.9936	502.704	13.0041	50.0428	2.701312	1.11408	1.699342
161.9935	491.529	7.00121	46.0328	2.691549	0.845173	1.663067
164.9934	515.77	3.00052	17.0078	2.712456	0.477197	1.230648
167.9933	485.498	8.00261	38.0217	2.686187	0.903232	1.580032
170.9932	483.438	8.00179	29.018	2.684341	0.903187	1.462667
173.993	486.488	5.00137	26.0148	2.687072	0.699089	1.41522
176.9929	516.907	8.00195	20.0098	2.713412	0.903196	1.301243
179.9928	483.517	11.0024	20.0106	2.684412	1.041487	1.30126
182.9927	468.319	7.00135	22.0107	2.670542	0.845182	1.342634
185.9926	447.127	3.00034	20.009	2.650431	0.47717	1.301225
188.9924	433.034	6.00232	22.0105	2.636522	0.778319	1.34263
191.9923	445.063	5.00097	24.0134	2.648421	0.699054	1.380454
194.9922	469.3	6.00147	20.0077	2.671451	0.778258	1.301197
197.9921	499.642	3.00046	22.0112	2.698659	0.477188	1.342644

Supplementary Figure 9 – ToF-SIMS Depth profile of pHEMA hydrogel

Sputter Time (s)	Normalised to total counts			
	Si+	C_4H_6N_2+	O_6H_13+	C_6H_9O_2+
	27.9788	82.0455	91.688	113.068
	Intensity	Intensity	Intensity	Intensity
0	3.00046	372.669	326.248	284.769
3	4.00147	316.333	908.534	3995.44
6	1.00034	372.981	1104.49	4585.98
9	2.00024	324.9	1134.22	4556.33
12	1.00027	318.247	1118.5	4572.45
15	2.00037	322.019	1153.36	4574.38
18	3.00082	260.218	1107.87	4448.37
21	0	325.27	1154.69	4492.75
24	0	307.236	1160.01	4683.3
27	1.00009	293.877	1188.91	4508.29
30	1.00003	321.455	1109.31	4411.83
33	0	311.466	1141.87	4254.09
36	0	305.037	1274.27	4324.66
39	2.00079	321.622	1222.72	4678.31
42	1.00015	286.062	1179.57	4521.26
45	2.00037	257.76	1148.98	4345.48
48	0	283.755	1167.52	4250.64
51	0	267.168	1189.87	4453.1
54	1.00027	275.14	1167.6	4490.7
57	1.00027	286.662	1265.24	4408.12
60	0	272.158	1183.36	3954.56
63	1.00027	234.943	1154.68	4012.67
66	1.00015	295.895	1185.41	4031.03
69	2.00037	248.662	1253.48	4219.84
72	0	250.915	1212.66	3931.81
75	3.00101	205.463	1177.92	3726.4
78	1.00009	238.699	1203.29	3636.35
81	3.00119	245.794	1168.52	3471.09
84	3.00064	239.599	1110.55	3590.37

87	2.00049	211.3	1105.85	3098.74	0.301136	2.324899	3.043696	3.491185
90	0	173.823	1047.32	2664.04	#NUM!	2.240107	3.020079	3.425541
93	1.00015	196.054	1066.25	2694.56	6.51E-05	2.292376	3.027859	3.430488
96	1.00015	183.95	1020.26	2568.21	6.51E-05	2.2647	3.008711	3.409631
99	2.00055	163.787	1112.85	2643.26	0.301149	2.214279	3.046437	3.42214
102	0	178.056	1044.74	2298.1	#NUM!	2.250557	3.019008	3.361369
105	2.00037	175.164	974.735	2255.95	0.30111	2.243445	2.988887	3.353329
108	3.00064	191.604	976.429	2863.22	0.477214	2.282405	2.989641	3.456855
111	354.88	136.01	603.538	2095.93	2.550082	2.133571	2.780705	3.321377
114	453.351	17.0319	85.5616	368.057	2.656435	1.231263	1.932279	2.565915
117	343.662	2.00122	29.0754	94.3424	2.536132	0.301295	1.463526	1.974707
120	341.598	5.00321	16.0245	79.2299	2.533515	0.699249	1.204784	1.898889
123	329.531	2.00085	16.0166	41.0815	2.517896	0.301215	1.20457	1.613646
126	423.975	3.00137	7.00663	17.0119	2.62734	0.47732	0.845509	1.230753
129	377.591	1.00015	3.00284	23.0238	2.577022	6.51E-05	0.477532	1.362177
132	459.579	3.00082	2.0011	21.0252	2.66236	0.47724	0.301269	1.32274
135	479.17	0	7.00706	12.0081	2.68049	#NUM!	0.845536	1.079474
138	523.787	1.00015	4.00403	21.025	2.719155	6.51E-05	0.602497	1.322736
141	560.687	0	3.00339	12.0117	2.74872	#NUM!	0.477612	1.079604
144	608.83	0	4.00293	16.0196	2.784496	#NUM!	0.602378	1.204652
147	565.074	1.00015	1.0007	10.008	2.752105	6.51E-05	0.000304	1.000347
150	606.299	3.00046	3.00357	16.0137	2.782687	0.477188	0.477638	1.204492
153	624.306	0	3.00247	6.0044	2.795398	#NUM!	0.477479	0.77847
156	646.116	1.00015	2.0022	6.00293	2.81031	6.51E-05	0.301507	0.778363
159	651.363	0	2.00122	6.0044	2.813823	#NUM!	0.301295	0.77847
162	642.312	1.00003	4.0044	2.0011	2.807746	1.3E-05	0.602537	0.301269
165	684.597	0	0	5.00229	2.835435	#NUM!	#NUM!	0.699169
168	713.061	1.00009	3.00266	2.00098	2.853127	3.91E-05	0.477506	0.301243
171	701.256	0	2.00085	2.00073	2.845877	#NUM!	0.301215	0.301188
174	704.61	0	3.00302	3.00113	2.847949	#NUM!	0.477558	0.477285
177	736.265	0	2.00208	2.00073	2.867034	#NUM!	0.301481	0.301188
180	741.271	0	0	7.00492	2.869977	#NUM!	#NUM!	0.845403
183	776.872	0	0	3.00137	2.890349	#NUM!	#NUM!	0.47732
186	751.56	0	0	1.00058	2.875964	#NUM!	#NUM!	0.000252
189	752.984	1.00009	2.00134	4.00318	2.876786	3.91E-05	0.301321	0.602405
192	802.131	0	0	3.00156	2.904245	#NUM!	#NUM!	0.477347
195	766.001	0	2.00147	2.0011	2.884229	#NUM!	0.301349	0.301269
198	749.793	0	0	2.00073	2.874941	#NUM!	#NUM!	0.301188
201	761.231	0	1.00113	1.00064	2.881516	#NUM!	0.00049	0.000278
204	817.959	0	0	3.00119	2.912732	#NUM!	#NUM!	0.477293
207	722.568	0	1.00113	1.00052	2.858879	#NUM!	0.00049	0.000226
210	826.328	0	2.00196	2.00098	2.917152	#NUM!	0.301455	0.301243
213	869.714	0	0	1.00021	2.939376	#NUM!	#NUM!	9.12E-05
216	821.168	0	3.00357	1.00076	2.914432	#NUM!	0.477638	0.00033

Supplementary figure 10 – ToF-SIMS depth profile of pHEMA hydrogel incubated in lysozyme

	Si+	C_4H_6N_2+	O_6H_13+	C_6H_9O_2+	Si+	C_4H_6N_2+	O_6H_13+	C_6H_9O_2+
	27.9753	82.0426	109.0704	113.0662	27.0232	81.0312	91.055	109.0704
Sputter Time (s)	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity
3.2768	6.00101	339.515	79.1655	158.499	3.197598	2.563747	2.400711	1.898536
9.8304	7.00082	278.144	167.588	325.55	3.199843	2.559575	2.481807	2.224243
16.384	0	246.023	275.414	632.253	3.297189	2.554186	2.647817	2.439986
22.9376	4.00053	225.901	363.461	875.037	3.372895	2.585651	2.764286	2.560458
29.4912	2.00012	202.83	435.343	1125.33	3.397711	2.623098	2.870229	2.638832
36.0448	3.00057	177.689	455.76	1284.32	3.445424	2.640195	2.9191	2.658736
42.5984	1.00008	208.976	485.13	1356.59	3.466547	2.637834	2.957191	2.685858
49.152	1.00005	203.889	558.11	1411.14	3.46334	2.622371	2.944707	2.74672
55.7056	0	206.966	535.753	1527.65	3.465743	2.667915	2.964834	2.728965
62.2592	1.00002	202.916	576.367	1491.89	3.491364	2.597725	2.983483	2.760699
68.8128	1.00007	229.044	581.419	1566.48	3.486029	2.667018	2.94749	2.764489
75.3664	2.00018	205.979	544.887	1615.74	3.484167	2.685539	2.974183	2.736306

81.92	1.00007	193.901	572.459	1538.96	3.493377	2.641665	2.978914	2.757744
88.4736	1.00011	207.899	586.755	1516.78	3.489662	2.67911	2.955767	2.768457
95.0272	0	236.127	567.06	1593.23	3.498681	2.666157	2.964267	2.753629
101.5808	1.0001	200.951	573.346	1541.36	3.492783	2.650451	2.977999	2.758417
108.1344	1.00014	193.875	575.386	1582.9	3.487695	2.679123	2.988221	2.759959
114.688	1.00008	175.79	567.173	1588.19	3.498709	2.659409	2.993719	2.753716
121.2416	1.00007	200.901	550.797	1609.9	3.503904	2.681835	2.984017	2.740992
127.7952	2.00024	203.981	563.528	1586.1	3.494152	2.6753	2.97945	2.750915
134.3488	7.0008	217.013	549.061	1536.33	3.482312	2.652692	2.957383	2.739621
140.9024	22.006	177.841	565.131	1509.64	3.495007	2.675289	2.963775	2.752149
147.456	54.0239	200.845	544.985	1488.95	3.47006	2.64061	2.964088	2.736385
154.0096	121.128	166.683	482.802	1350.96	3.451158	2.60733	2.895665	2.683769
160.5632	274.6	147.492	398.86	1062.39	3.397192	2.564146	2.844471	2.60082
167.1168	517.127	115.267	278.281	721.808	3.295002	2.385354	2.695051	2.444484
173.6704	953.041	70.1058	177.532	432.171	3.149755	2.188563	2.4612	2.249277
180.224	1467.55	30.0167	62.072	148.282	2.929744	1.930138	2.224354	1.792896
186.7776	2008.81	11.0026	27.0126	47.0319	2.67005	1.415331	1.778781	1.431566
193.3312	2065.56	3.00048	8.00096	22.0071	2.342022	1.176279	1.518815	0.903142

Supplementary figure 11 – ToF-SIMS depth profile of phema/lysozyme film

Etch Time (s)	C 1s %	O 1s %	Si 2p %	C/O	O/C Measured	O/C Literature
0	62.6681	37.3319	0	1.678674	0.595708	0.666667
10	63.1732	36.8268	0	1.715414	0.58295	0.666667
20	61.3386	38.6614	0	1.586559	0.630295	0.666667
30	62.2394	37.7606	0	1.648263	0.606699	0.666667
40	61.3535	38.6465	0	1.587556	0.629899	0.666667
50	61.0077	38.9923	0	1.564609	0.639137	0.666667
60	62.6833	37.3167	0	1.679765	0.595321	0.666667
70	61.7245	38.2755	0	1.612637	0.620102	0.666667
80	61.4396	38.5604	0	1.593334	0.627615	0.666667
90	62.9455	37.0545	0	1.698728	0.588676	0.666667
100	63.6489	36.3511	0	1.750948	0.571119	0.666667
110	62.9493	37.0507	0	1.699004	0.58858	0.666667
120	63.504	36.496	0	1.740026	0.574704	0.666667
130	61.9591	38.0409	0	1.62875	0.613968	0.666667
140	60.8351	39.1649	0	1.553307	0.643788	0.666667
150	62.1143	37.8857	0	1.639518	0.609935	0.666667
160	62.2173	37.7827	0	1.646714	0.60727	0.666667
170	63.6677	36.3323	0	1.752372	0.570655	0.666667
180	61.7973	38.2027	0	1.617616	0.618194	0.666667
190	62.4249	37.5751	0	1.661337	0.601925	0.666667
200	59.7526	40.2474	0	1.484633	0.673567	0.666667
210	64.1339	35.8661	0	1.788148	0.559238	0.666667
220	61.6704	38.3296	0	1.60895	0.621523	0.666667
230	59.8624	40.1376	0	1.491429	0.670498	0.666667
240	58.5383	36.3915	5.07022	1.608571	0.62167	0.666667
250	59.7495	37.1631	3.08741	1.607764	0.621982	0.666667
260	57.3374	39.1476	3.51494	1.464647	0.682759	0.666667
270	56.5445	37.1255	6.32998	1.523064	0.656571	0.666667
280	53.5804	35.9066	10.5131	1.492216	0.670144	0.666667
290	47.2183	37.4734	15.3083	1.260048	0.79362	0.666667
300	38.8619	40.3125	20.8255	0.964016	1.037327	0.666667
300	47.7974	35.8427	16.3599	1.333532	0.749888	0.666667
310	37.9541	37.6808	24.3651	1.007253	0.992799	0.666667
320	34.2465	36.8927	28.8608	0.928273	1.077269	0.666667
330	22.3295	35.5684	42.1021	0.62779	1.592888	0.666667
340	13.7045	35.7604	50.5351	0.383231	2.609391	0.666667
350	22.9465	31.0516	46.0019	0.73898	1.353217	0.666667

360	6.39568	31.6832	61.9211	0.201863	4.953844	0.666667
370	6.91179	29.7562	63.332	0.232281	4.305137	0.666667

Supplementary figure 12 – XPS depth profile of PLGA

Etch Time	C 1s %	O 1s %	Si 2p %	O/C measured	Theoretical (calculated)	Literature (castner)			
						C1s	O1s	O/C	% ice
0	27.8449	72.1551	0	2.591322	0.5	66.7	33.3	0.49925	38.8551
120	26.6355	73.3645	0	2.754388	0.5	66.7	33.3	0.49925	40.0645
240	32.2917	67.7083	0	2.096771	0.5	66.7	33.3	0.49925	34.4083
360	43.1956	56.8044	0	1.315051	0.5	66.7	33.3	0.49925	23.5044
480	48.3112	51.6888	0	1.069913	0.5	66.7	33.3	0.49925	18.3888
600	54.5233	45.4767	0	0.834078	0.5	66.7	33.3	0.49925	12.1767
720	60.0604	39.9396	0	0.664991	0.5	66.7	33.3	0.49925	6.6396
840	61.4219	38.5781	0	0.628084	0.5	66.7	33.3	0.49925	5.2781
960	64.8658	35.1342	0	0.541644	0.5	66.7	33.3	0.49925	1.8342
1080	66.1191	33.8809	0	0.512422	0.5	66.7	33.3	0.49925	0.5809
1200	66.3494	33.6506	0	0.507173	0.5	66.7	33.3	0.49925	0.3506
1320	68.3007	31.6993	0	0.464114	0.5	66.7	33.3	0.49925	-1.6007
1440	67.8066	32.1934	0	0.474783	0.5	66.7	33.3	0.49925	-1.1066
1560	68.4821	31.5179	0	0.460236	0.5	66.7	33.3	0.49925	-1.7821
1680	68.5837	31.4163	0	0.458072	0.5	66.7	33.3	0.49925	-1.8837
1800	68.0091	31.9909	0	0.470391	0.5	66.7	33.3	0.49925	-1.3091
1920	67.0872	32.9128	0	0.490597	0.5	66.7	33.3	0.49925	-0.3872
2040	67.5759	29.9659	2.45819	0.443441	0.5	66.7	33.3	0.49925	-3.3341
2160	67.2466	30.2327	2.52071	0.44958	0.5	66.7	33.3	0.49925	-3.0673
2280	66.0575	30.5639	3.37856	0.462686	0.5	66.7	33.3	0.49925	-2.7361
2400	63.8445	29.8157	6.3398	0.467005	0.5	66.7	33.3	0.49925	-3.4843
2520	61.8645	30.3131	7.82238	0.489992	0.5	66.7	33.3	0.49925	-2.9869
2640	59.226	28.5976	12.1764	0.482856	0.5	66.7	33.3	0.49925	-4.7024
2760	57.1049	27.7129	15.1821	0.485298	0.5	66.7	33.3	0.49925	-5.5871
2880	54.5793	27.1927	18.228	0.498224	0.5	66.7	33.3	0.49925	-6.1073
3000	50.5687	25.6642	23.7671	0.507512	0.5	66.7	33.3	0.49925	-7.6358
3120	48.0814	24.3845	27.5341	0.50715	0.5	66.7	33.3	0.49925	-8.9155
3240	45.4402	22.6706	31.8892	0.498911	0.5	66.7	33.3	0.49925	-10.6294

Supplementary Figure 13 - pHEMA table displaying values from through thickness XPS profile

Etch Time	C 1s %	N 1s %	O 1s %	Si 2p %	Lysozyme theoretical	O/C measured	O/C theoretical	N/O measured	N/O theoretical
0	35.42	6.40	58.18	0.00	1.00	1.64243	0.60	0.109936	0.01
120	27.15	3.68	69.17	0.00	1.00	2.547448	0.60	0.053136	0.01
240	30.19	4.04	65.77	0.00	1.00	2.178402	0.60	0.061431	0.01
360	33.65	3.49	62.86	0.00	1.00	1.86834	0.60	0.055555	0.01
480	37.26	2.30	58.44	0.00	1.00	1.56862	0.60	0.039319	0.01
600	40.93	2.53	56.53	0.00	1.00	1.381075	0.60	0.044781	0.01
720	38.98	3.23	57.78	0.00	1.00	1.482212	0.60	0.055946	0.01
840	45.03	1.57	54.40	0.00	1.00	1.208007	0.60	0.028927	0.01
960	47.77	1.57	50.66	0.00	1.00	1.060595	0.60	0.031064	0.01
1080	45.65	2.47	51.89	0.00	1.00	1.136784	0.60	0.047529	0.01
1200	50.50	2.47	49.50	0.00	1.00	0.980115	0.60	0.049825	0.01
1320	50.23	1.67	48.09	0.00	1.00	0.957361	0.60	0.034783	0.01
1440	51.51	1.75	46.74	0.00	1.00	0.907296	0.60	0.03742	0.01
1560	48.88	1.57	45.08	2.47	1.00	0.922287	0.60	0.034772	0.01
1680	48.53	1.63	48.15	2.69	1.00	0.992188	0.60	0.033873	0.01
1800	49.66	1.18	43.43	2.73	1.00	0.874584	0.60	0.027221	0.01
1920	48.67	1.42	46.55	3.36	1.00	0.956466	0.60	0.030421	0.01
2040	46.76	1.38	45.79	6.07	1.00	0.979255	0.60	0.030045	0.01
2160	49.94	0.15	43.52	6.39	1.00	0.871458	0.60	0.003431	0.01
2280	48.64	0.29	44.19	5.88	1.00	0.90857	0.60	0.006455	0.01
2400	44.68	0.45	42.64	10.23	1.00	0.954474	0.60	0.010653	0.01

Supplementary figure 14 – pH EMA hydrogel incubated in lysozyme (1)

Etch Time	C 1s %	N 1s %	O 1s %	Si 2p %	Si 2s %	o/c measured	O/C theoretical	N/O measured	N/O theoretical	Interface gel
0	54.7	11.9	33.2	0	0	0.606	0.6	0.36125	0.01	3600
240	14.9	4.81	80.2	0	0	5.373	0.6	0.05998	0.01	3600
480	19.3	6.12	74.5	0	0	3.85952	0.6	0.08215	0.01	3600
720	28.4	3.86	64.6	0	0	2.27158	0.6	0.05977	0.01	3600
960	31.0	2.53	63.4	0	0	2.04140	0.6	0.04005	0.01	3600
1200	39.2	2.17	56.6	0	0	1.44364	0.6	0.03841	0.01	3600
1440	45.4	1.68	52.7	0	0	1.16366	0.6	0.03194	0.01	3600
1680	54.0	1.52	44.4	0	0	0.82107	0.6	0.03430	0.01	3600
1920	57.0	1.88	42.0	0	0	0.73588	0.6	0.04478	0.01	3600
2160	57.1	1.67	42.1	0	0	0.73845	0.6	0.03964	0.01	3600
2400	52.2	1.67	41.7	2.48	3.474	0.79998	0.6	0.04002	0.01	3600
2640	44.6	1.52	45.4	4.94	4.6382	1.01790	0.6	0.03357	0.01	3600
2880	36.7	1.42	48.5	6.78	7.7840	1.32102	0.6	0.02938	0.01	3600
3120	29.7	1.76	52.5	9.05	8.6177	1.76672	0.6	0.03360	0.01	3600
3360	27.0	1.56	50.7	10.5	11.048	1.87468	0.6	0.03085	0.01	3600
3600	22.4	1.36	51.2	11.9	14.313	2.28290	0.6	0.02665	0.01	3600
3840	21.4	1.56	51.9	14.0	12.533	2.42319	0.6	0.03013	0.01	3600
4080	20.2	1.31	52.6	13.1	13.557	2.59541	0.6	0.02498	0.01	3600
4320	18.4	0.91	52.7	14.7	14.054	2.86330	0.6	0.01733	0.01	3600
4560	14.1	0.81	55.5	15.2	15.094	3.92458	0.6	0.01466	0.01	3600
4800	12.8	0.45	55.1	15.7	15.861	4.29402	0.6	0.00826	0.01	3600
5040	11.9	0	55.1	17.8	15.7074	4.631369	0.6	0	0.01	3600
5280	8.68	0	56.4	17.2	17.6369	6.501628	0.6	0	0.01	3600

Supplementary figure 15 - pHEMA hydrogel incubated in lysozyme (1)

Counter	Time	C 1s %	N 1s %	O 1s %	O/C ratio	O/N	N/C
0	0	64.79489	13.90649	21.29862	0.328708	1.53156	42.30647
1	12	65.71081	13.01701	21.27218	0.323724	1.634183	40.21018
2	24	65.47236	13.64011	20.88752	0.319028	1.531331	42.7552
3	36	65.28049	13.10723	21.61228	0.331068	1.648882	39.59075
4	48	64.59216	13.06573	22.34211	0.345895	1.709978	37.77368
5	60	64.2646	13.00221	22.73318	0.353743	1.748409	36.75605
6	72	63.57861	12.79942	23.62197	0.37154	1.84555	34.44968
7	84	63.8338	12.35992	23.80628	0.372942	1.926087	33.1417
8	96	63.33302	12.2274	24.43958	0.38589	1.998755	31.68623
9	108	63.78367	12.13498	24.08135	0.377547	1.984457	32.14162
10	120	62.25971	12.42806	25.31223	0.406559	2.0367	30.56892
11	132	61.59725	12.45481	25.94794	0.421252	2.083367	29.5662
12	144	60.76132	12.76837	26.47031	0.435644	2.073116	29.30918
13	156	61.74509	11.38263	26.87228	0.435213	2.360815	26.15415
14	168	60.61232	11.73741	27.65026	0.456182	2.355738	25.72966
15	180	60.45775	11.41337	28.12889	0.465265	2.464556	24.53089

Supplementary figure 16 – XPS damage study

C-C CPS (eV)	C-O-C CPS (eV)	O-C=O CPS (eV)	C-C (%)	C-O-C (%)	O-C=O (%)
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0	2960.75	1081.96	1014.66	58.5433	21.3937	20.063
12.85714	2917.82	1197.44	1006.78	56.9659	23.3782	19.6559
25.71429	2890.66	1193.04	884.649	58.1815	24.0128	17.8057
38.57143	2833.13	1369.41	928.485	55.2157	26.6888	18.0955
51.42857	2995.77	1047.29	957.697	59.9063	20.9427	19.151
64.28571	2841.84	1207.55	913.304	57.2641	24.3325	18.4034
77.14286	2887.45	1225.5	891.872	57.6934	24.4864	17.8203
90	2965.97	1074.32	891.722	60.1372	21.7825	18.0803
102.8571	2864.38	1084.31	900.457	59.0697	22.3609	18.5694
115.7143	2799.47	1182.95	826.628	58.2126	24.5984	17.189
128.5714	2911.97	1048.89	858.267	60.4252	21.7652	17.8096
141.4286	2639.02	1263.24	793.288	56.2026	26.9029	16.8945
154.2857	2924.45	991.605	901.676	60.7018	20.5824	18.7158
167.1429	2733.59	1358.52	834.738	55.4835	27.5738	16.9426
180	2706.24	1128.49	793.756	58.4692	24.3814	17.1494

Supplementary figure 17 – Change in c1s peak contribution from damage study

	total	Si+	C_4H_6N_2+	O_2H_5+	C_6H_9O_2+	C_4H_6N_2	O_2H_5+	C_6H_9O_2+
	N/A	27.9765	82.0455	37.0294	113.0675	82.0455	37.0294	113.0675
#Data Point #	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	Intensity	
1	112395	9	374	3347	1409	0.003328	0.029779	0.012536
2	112935	13	394	3407	1254	0.003489	0.030168	0.011104
3	113456	5	340	3380	1198	0.002997	0.029791	0.010559
4	113684	7	342	3586	1083	0.003008	0.031544	0.009526
5	111025	3	324	3554	1044	0.002918	0.032011	0.009403
6	110297	0	332	3906	943	0.00301	0.035413	0.00855
7	107994	3	328	3930	841	0.003037	0.036391	0.007787
8	103764	6	294	4092	798	0.002833	0.039436	0.007691
9	103443	6	301	4185	770	0.00291	0.040457	0.007444
10	101737	5	273	4135	722	0.002683	0.040644	0.007097
11	99064	3	315	4122	684	0.00318	0.041609	0.006905
12	97579	4	245	4221	656	0.002511	0.043257	0.006723
13	94940	6	257	4264	605	0.002707	0.044913	0.006372
14	92009	4	240	4116	571	0.002608	0.044735	0.006206
15	91106	8	268	4160	554	0.002942	0.045661	0.006081
16	89939	6	232	4144	514	0.00258	0.046076	0.005715
17	87713	2	225	4133	491	0.002565	0.04712	0.005598
18	87159	3	212	4097	483	0.002432	0.047006	0.005542
19	85473	2	203	4087	448	0.002375	0.047816	0.005241
20	85275	5	187	4148	461	0.002193	0.048643	0.005406
21	83847	3	209	4075	383	0.002493	0.0486	0.004568
22	82305	5	190	3989	411	0.002308	0.048466	0.004994
23	80783	3	181	3990	354	0.002241	0.049392	0.004382
24	80937	2	173	3988	367	0.002137	0.049273	0.004534
25	79581	3	161	3899	360	0.002023	0.048994	0.004524
26	79768	4	168	3976	330	0.002106	0.049845	0.004137
27	80079	4	172	4001	334	0.002148	0.049963	0.004171
28	77190	2	153	3850	312	0.001982	0.049877	0.004042
29	77664	2	164	3903	278	0.002112	0.050255	0.00358
30	76813	5	159	3816	294	0.00207	0.049679	0.003827
31	76836	3	142	3967	257	0.001848	0.051629	0.003345
32	76955	8	154	3823	256	0.002001	0.049678	0.003327
33	76705	5	164	3872	272	0.002138	0.050479	0.003546
34	76920	2	157	3876	241	0.002041	0.05039	0.003133
35	76123	1	141	3870	252	0.001852	0.050839	0.00331

36	74908	3	160	3767	267	0.002136	0.050288	0.003564
37	74863	4	133	3887	251	0.001777	0.051922	0.003353
38	74531	4	118	3796	222	0.001583	0.050932	0.002979
39	73372	3	143	3746	238	0.001949	0.051055	0.003244

Supplementary figure 18 – ToF-SIMS damage study

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