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Correlating drug release with microstructure of hydrogel-based delivery systems

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Abstract

Hydrogels are three-dimensional networks of polymers which can absorb a large amount of water. They have been utilised in pharmaceutical and biomedical applications ranging from an established application in oral dosage tablets to more recent use in regenerative medicines. The use of hydrogels for oral sustained drug delivery has been demonstrated in numerous previous studies where hydroxypropyl methylcellulose (HPMC) was employed to reduce dosing frequency, thereby improving patient compliance. In previous research the focus of HPMC studies was limited to its effects on drug release. Injectable hydrogels have recently received increased attention as potential tissue regeneration platforms. Due to their high water content, hydrogels can be used to replicate the microenvironment of the cartilage extracellular matrix. In both systems, however, the correlation of formulation variables and release performance is still poorly understood. The current research aims to provide insight into the release mechanism of active substances from two hydrogel-based delivery systems, tablets and injectable hydrogels, using chemical and electron imaging techniques.

A series of caffeine tablets containing various amounts of HPMC and particle size fractions were produced and cross-sectioned. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used to analyse the caffeine tablets and their raw materials. ToF-SIMS successfully distinguished key components and revealed the characteristics of compressed ingredients in tablets. Changing HPMC factors significantly affected the microstructure of the tablets. The inter-particle distance and intersection counts of HPMC were proved to be indicative of HPMC's role in developing gel layers during the early dissolution periods (< 60 minutes). Prediction of caffeine release from the HPMC matrix tablets was made with the maximum error of less than 5 %. Using ToF-SIMS, the interconnectivity of HPMC networks in 30 %w/w HPMC tablet matrices was evident which is in agreement with percolation theory. This work additionally conducted an examination of microstructural changes to the polymer concentration after exposure to water. After 15 minutes dissolution, the gel layer was completely formed in the 30 %w/w HPMC tablets, whereas, the incomplete

gel layer was found when using 15 %w/w HPMC. The caffeine particles were only retained in the core of the tablets with the complete gel layer functioning as a depot for sustained drug release. Considerable insight has been gained to elucidate the release performance of drug from HPMC matrix tablets which might be useful for improving knowledge about the performance of other extended release systems.

In the case of injectable hydrogels, the incorporation of human platelet lysate (PL) was investigated relating to the application of hydrogels for protein delivery in cartilage regeneration. *In situ* forming hydrogels composed of hyaluronic acid-tyramine (HA-TA) and dextran tyramine (Dex-TA) were prepared by an enzymatically crosslinking reaction with horseradish peroxidase and H₂O₂. The gels have been optimised by varying the concentration of HA-TA and Dex-TA, H₂O₂/TA molar ratio and PL/PBS ratio to obtain fast gelation time, good handleability and good mechanical properties of the gels. The Dex-TA gels exhibited faster protein release than the HA-TA gel. Cryo-focused ion beam scanning electron microscopy and a sublimation technique were used to obtain and image the internal structure of hydrogels by preserving them close to their native hydrated state. The ultrastructure of the gels was found to be related to the release rate of proteins. The faster release of growth factors and cytokines from the Dex-TA gels can be explained by the higher porosity and larger pores of the Dex-TA gels. On the other hand, the pores of the HA-TA gels were found to be uniformly smaller which may lead to the more delayed release of proteins.

This research has investigated the formulation-structure-release relationship of hydrophilic matrices and *in situ* forming injectable hydrogels. Based on this knowledge, future work may focus on designing hydrogel-based delivery systems and tailoring drug release. Therefore, it could offer significant development for effective formulation designs.

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Abbreviations

API	Active pharmaceutical ingredient
Ar-GCIB	Argon gas cluster ion beam
ATR	Attenuated total reflection
BCS	Biopharmaceutical classification system
BEs	Backscattered electrons
BSA	Diluted albumin
b-FGF	Basic fibroblast growth factor
CCD	Charge-coupled devices
CI	Confidence interval
CLSM	Confocal laser scanning microscopy
CMC	Carboxymethyl cellulose
CR	Controlled release
CRM	Confocal Raman microscopy
Cryo-FIB-SEM	Cryo-focused ion beam scanning electron microscopy
Cryo-3D OrbiSIMS	Cryo-three dimensional Orbitrap secondary ion mass spectrometry
Dex-TA	Dextran-tyramine
DS	Degree of substitution
ECM	Extracellular matrix
EGF	Epidermal growth factor
FDA	Food and Drug Administration
FIBs	Focused ion beams
FIR	Far-infrared
FTIR	Fourier-transform infrared spectroscopy
GI	Gastrointestinal
GPO	Government Pharmaceutical Organization
GRAS	Generally regarded as safe
G'	Storage modulus
G''	Loss modulus
HA-TA	Hyaluronic acid-tyramine
HDPE	High density polyethene

HGF	Hepatocyte growth factor
HPL	Human platelet lysate
HPLC	High performance liquid chromatography or high pressure liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
INN	International nonproprietary name
IR	Immediate release
LMIG	Liquid metal ion gun
LMIS	Liquid metal ion source
MAE	Mean absolute error
MALDI	Matrix assisted laser desorption/ionisation
MC	Methyl cellulose
MCC	Microcrystalline cellulose
MDT	Mean dissolution time
MEC	Minimum effective concentration
MgSt	Magnesium stearate
MLR	Multiple linear regression
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
MTC	Minimum toxic concentration
m/z	Mass-to-charge ratio
NDAs	New drug applications
NIR	Near infrared
NMR	Nuclear magnetic resonance
NMRC	Nanoscale and Microscale Research Centre
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
PDGF	Platelet-derived growth factor
PDMS	Polydimethylsiloxane
PL	Platelet lysate
PS	Polystyrene

PVP	Polyvinylpyrrolidone
QbD	Quality by design
r	Pearson correlation coefficient
R^2	Coefficient of determination
$\bar{R}^2$	Adjusted coefficient of determination
RSD	Relative standard deviation
SEM	Scanning electron microscopy
SEs	Scanning electrons
SIMS	Secondary ion mass spectrometry
SR	Sustained release
TEM	Transmission electron microscopy
TGF- β	Transforming growth factor- β
ToF	Time-of-flight
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
USP	United States Pharmacopeia
UV-vis	Ultraviolet-visible spectroscopy
VEGF	Vascular endothelial growth factor
XPS	X-ray photoelectron spectroscopy

Chapter 1:

Introduction

1.1 Introduction to hydrogel based pharmaceutical systems

Hydrogels are three dimensional, water-swollen, cross-linked polymeric networks of hydrophilic polymers.¹⁻³ Hydrogels can be classified into natural and synthetic hydrogels, based on their origins. The natural sources of hydrogels include proteins (e.g. collagen)¹, polysaccharides (e.g. hyaluronic acid, starch, alginate).¹ Examples of synthetic materials are poly(ethylene oxide), poly(acrylic acid), poly(propylene fumarate-co-ethylene glycol), and polypeptides.² Due to their nature in imbibing large amounts of water hydrogels can exhibit closely similar properties to natural tissue. They can also be tailored to controllable responses in order to shrink or expand with physical or chemical stimuli such as temperature, light, pressure, pH and enzymes.¹

With their adjustable biodegradability, porous structure and good mechanical properties, hydrogels serve as an interesting platform to deliver drug and protein delivery for the therapy of several diseases through multiple routes of administration. The cross-links between the polymer chains of hydrogels can develop the resistance to dissolution and facilitate versatile controlled release delivery applications. They are diverse from conventional oral dosage forms to more advanced targeted drug delivery systems. This research focuses on the use of hydrogel-based delivery systems for two main purposes, oral pharmaceutical tablets and injectable hydrogels.

1.2 Hydrogel based extended release tablets

Tablets are solid pharmaceutical dosage forms and the mainstay of pharmacotherapy. They provide numerous advantages to both patients and pharmaceutical companies due to simplicity and low-cost manufacturing, good stability and acceptable palatability. Tablets are diverse in different types depending on manufacturing approach and medical uses, e.g. plain tablets, coated tablets, multi-layer tablets, effervescent tablets, chewable tablets, buccal tablets and vaginal tablets for example.⁴⁻⁵ They are mainly administered orally and designed for systemic action.⁶

1.2.1 Extended release tablets

After an oral tablet has been administered into the body, the tablet will travel to the gastrointestinal (GI) tract. There are steps involving what the body will do to a drug (i.e. pharmacokinetics). An active pharmaceutical ingredient (API) is liberated from the dosage form by which the tablet may disintegrate into smaller particles. The API is dissolved into solution in the GI fluid and absorbed either via passive or active transport. It is vital to achieving optimum drug concentration at the site of action for effective therapeutic outcomes. Plasma drug concentration is commonly used to refer to the drug concentration at the target site. The drug is later metabolised by enzymes, most commonly cytochrome P450 enzymes, and excreted from the body.

Figure 1.1 illustrates plasma drug concentration-time profiles of immediate release (IR), sustained release (SR) and controlled release (CR) tablets. The therapeutic window is a range between the minimum effective concentration (MEC) and the minimum toxic concentration (MTC). If the concentration of the drug in plasma is below the MEC, it has a high probability of being subtherapeutic. On the other hand, if plasma concentration is above the MTC, adverse effects or toxicity may occur. In order to attain efficient treatment, drug concentration needs to maintain in the therapeutic window. This window could be narrow or broad, depending on the safety and effectiveness of the drug. For conventional/IR systems, the quantity of the API in the bloodstream is dropped

rapidly after the drug is metabolised and eliminated. Therefore, the IR dosage forms require repeated dosing for effective outcomes. Consequently, the fluctuation of the plasma concentration due to the IR systems is unavoidable. However, the concentration of drug in plasma is critical. For narrow therapeutic index drugs (e.g. anti-cancer drugs), the adverse effect or toxicity possibly occurs due to the abrupt rise of drug concentration. Frequent dosing also leads to poor patient adherence to the regimen and thereby unsuccessful treatment.

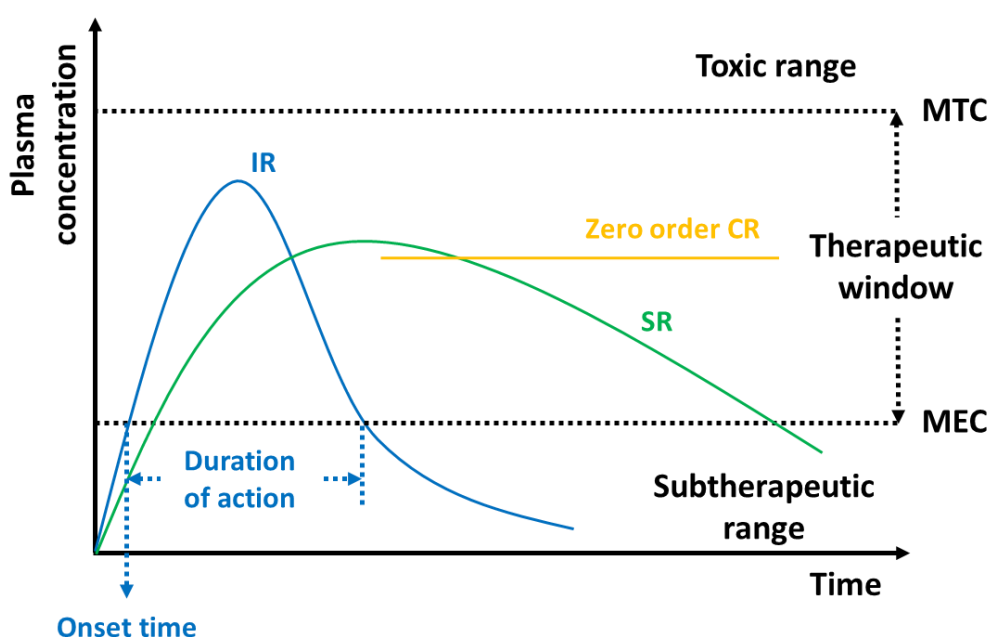


Figure 1.1. Schematic pharmacokinetic profiles of immediate release (IR), sustained release (SR) and controlled release (CR) dosage forms showing the therapeutic window which is the range between the minimal effective concentration (MEC) and minimal toxic concentration (MTC), adapted from Rosenbaum (2016)⁷.

The modification of the release aims to improve the efficacy and safety of the therapeutics by means of controlling the rate, time or place of the drug in the body. There are terms relating to modified release systems which can be defined.⁸ For example, delayed release is when the API is not promptly released after initial administration. Targeted release systems attempt to control the distribution of the drug in the body. Extended release systems can be categorised into sustained release and controlled release systems. SR tablets aim to maintain release rate over a sustained period. CR systems provide a sustained release

profile with constant plasma concentrations and typically follow zero-order kinetics. In general, the advantages of the extended release systems over conventional release formulations are slowing down the release of highly water-soluble drugs, reducing dosing frequency, improving patient compliance and minimising dose-dependent side effects.⁶ Table 1.1 lists several advantages offered by the extended release formulations and their challenges.

Table 1.1. Common advantages and disadvantages of extended release dosage forms adapted from Tiwari and Rajabi-Siahboomi (2008).⁹

Advantage/Disadvantage		Outcome
Advantage		
Extended/sustained action	• Reduction in dosing frequency • Reduction in total medication use • Improvement in patient compliance	
The reduced fluctuation of drug level	• Reduction of dose-related adverse effects/toxicity • Stabilisation of medical condition	
Limitation/Disadvantage		
Increased possibility of dose dumping due to poor manufacturing	• Increased risk of adverse effects/toxicity	
Difficulty in dosing adjustment	• Inefficiency in treatment • Increased risk of adverse effects/toxicity	
Crushing/splitting not allowed e.g. reservoir type systems	• Trouble in dosing adjustment • Trouble in enteral feeding	

1.2.2 Hydrophilic matrices

A hydrophilic matrix is a matrix system of diffusion-based sustained release dosage forms. It is a homogeneous dispersion of drugs within the skeleton of one or more hydrophilic polymers. The hydrophilic polymers can be either natural or derived from synthetic sources. The examples of hydrophilic polymers are

cellulose derivatives (e.g. hydroxypropyl methylcellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, methylcellulose, ethylcellulose, and cellulose acetate phthalate), sodium alginate, xanthan gum, polyethylene oxide and carbomer.¹⁰ Drug release from the hydrophilic matrix generally does not follow zero-order kinetics.⁸ Hydrophilic matrices have several benefits. They are appropriate for different active substances and excipients which have various properties. Drug loading is adjustable. The manufacturing process is short and cost-effective as they just require conventional manufacturing process and instruments. As the manufacturing process is simple, the release kinetics and profiles can be easily modified. When compared to other systems such as reservoir systems, there is no risk of dose dumping.⁸

1.2.3 Hydroxypropyl methylcellulose

Hydroxypropyl methylcellulose (HPMC; INN hypromellose) is a semi-synthetic derivative of cellulose.¹¹⁻¹² It is an odourless and tasteless, white or creamy-white fibrous or granular powder.¹³ HPMC is a linear polymer with the backbones of cellulose with a repeating structure of etherified anhydroglucose unit. The chemical structure of the HPMC is illustrated in Figure 1.2. It can be seen that the substituents R could be either $-\text{CH}_3$, $-\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$ or $-\text{H}$ group. There are different types of HPMC according to the degree of methoxy ($-\text{OCH}_3$) or hydroxypropoxy ($-\text{OCH}_2\text{CH}(\text{OH})\text{CH}_3$) groups.¹⁴ The HPMC classification code is specified by the United States Pharmacopeia (USP)¹⁵ using a four-digit number shown in Table 1.2. The first two digits represent the approximate percentage content of methoxyl group, whereas, the last two digits correspond to the approximate percentage content of hydroxypropoxyl substitution, calculated on the dried basis.¹⁵ Table 1.2 shows the limits of each HPMC type classified by the USP.

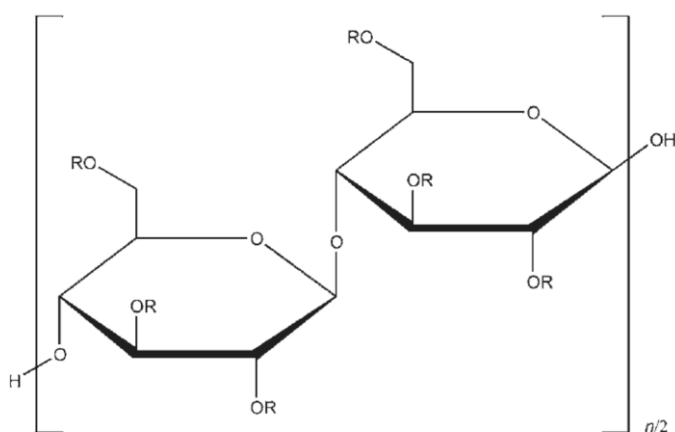


Figure 1.2. Chemical structure of HPMC polymer, where R is H, CH₃ or CH₃CH(OH)CH₂.¹³

Table 1.2. Specification of hypromellose in USP 32¹⁵ and examples of its commercially available products.

Substitution types	Methoxy (%)		Hydroxypropyl (%)		Commercial examples
	Min.	Max.	Min.	Max.	
1828	16.5	20.0	23.0	32.0	J*
2208	19.0	24.0	4.0	12.0	K100 LV ^{*,‡} , K4M ^{*,‡} , K15M ^{*,‡} , K100M ^{*,‡} , 90SH [†]
2906	27.0	30.0	4.0	7.5	F*, 65SH [†]
2910	28.0	30.0	7.0	12.0	E4M ^{*,‡} , E10M CR*, 60SH [†]

* METHOCEL products (Dow Chemical Company, US).

† METOLOSE products (Shin-Etsu Chemical Co., Japan).

‡ BENECEL products (Ashland, US)

As can be seen in Table 1.2 commercially available products of HPMC can be classified by codes given by the manufacturers. For example, the Dow Chemical Company assigns the letter in the first part of the METHOCEL codes to the degree of substitution. For example, HPMC 2208 USP is available in the form

of the K grades. As shown in Table 1.2, the K grades have a methoxy substitution in a range of 19.0 to 24.0% and a hydroxypropyl group in a range of 4.0 to 12.0%, calculated on the dried basis. The latter part of the code indicates the viscosity of the 2 % HPMC aqueous solution, calculated in millipascal-seconds (mPa·s) at 20 °C. The viscosity corresponds to the degree of polymerisation. The letter 'C' is mostly used to represent a multiplier of 100, and the letter 'M' is used to represent a multiplier of 1000 for the viscosity values. Other letters identify special grades of products such as 'P' denotes METHOCEL Premium products, 'LV' refers to special low-viscosity products and 'CR' for a controlled release grade. For instance, METHOCEL K4M refers to HPMC 2208 USP which has the viscosity of 4000 mPa·s at 2 % concentration in water at 20 °C.¹⁶

HPMC is one of the most frequently used polymers in the preparation of hydrophilic matrices. Until now, HPMC has been utilised as a matrix polymer in more than 100 controlled release products in the US market.¹⁷ HPMC can hydrate rapidly and produce a robust viscous gel which retards the influx of water and controls drug release.¹⁴ The viscosity of the HPMC gel is not affected by pH.¹⁸ It has high swelling capability. In term of formulation aspects, this polymer can be directly compressed.¹⁸ HPMC is non-ionic so that it is very versatile and compatible with electrolytes and several ingredients.¹⁸ Some of the excipients successfully used in combination with HPMC include pectin¹⁹, carboxymethyl cellulose sodium²⁰, ethylcellulose²⁰, xanthan gum²¹⁻²², alginate²³, PEG 6000²⁴. The safety of HPMC is also broadly accepted by the FDA as it is generally regarded as safe (GRAS).

1.2.4 Hydration, swelling and erosion of HPMC matrices

As mentioned in Section 1.2.2, HPMC is a swellable polymer which can hydrate and form a gelatinous layer. HPMC then plays an important role in preparations of sustained release tablets, especially hydrophilic matrices. In fact, extensive research on the release mechanism of the hydrophilic matrices has been studied for decades.²⁵ Drug release from the hydrophilic matrices is a complex process. However, the process of drug dissolution from the HPMC matrix tablets includes water penetration with no or partial HPMC gelation, complete gelation of HPMC

and erosion of HPMC²⁶ (Figure 1.3). When a dried HPMC matrix is in contact with an aqueous fluid, the hydration of the matrix occurs. By means of water uptake to the hydrophilic matrix, the components exposed to the water may start to dissolve or swell. A ‘burst’ release refers to the release of the drug molecules existing on the surface of the tablet. In the case of HPMC, its chains gradually unfold and extend due to the formation of hydrogen bonding between the sites on the HPMC chains and water molecules.¹² Water continues to diffuse into the system until there is a phase transition of the matrix from the glassy state to the rubbery state, leading to the formation of a gel-like layer. Consequently, the gel layer can act as a barrier to retard water penetration. As can be seen in Figure 1.3, the two boundaries of the gel layer can be observed: (1) erosion front and (2) swelling front. Erosion front is the position where the swollen matrix meets the solvent, whereas, the swelling front separates the glassy polymer from its rubbery state.²⁷ Moreover, another front inside the gel layer which locates at the edge between the area of dissolved and undissolved drugs was later described.²⁸ Details in the observation of the gel layer are further reviewed in Section 1.3.3. When the HPMC particles in the matrix fully hydrate, the gel is fully expanded, reaching to the highest thickness of the gel layer. The swellable gel layer is then finally eroded away until it is completely dissolved.

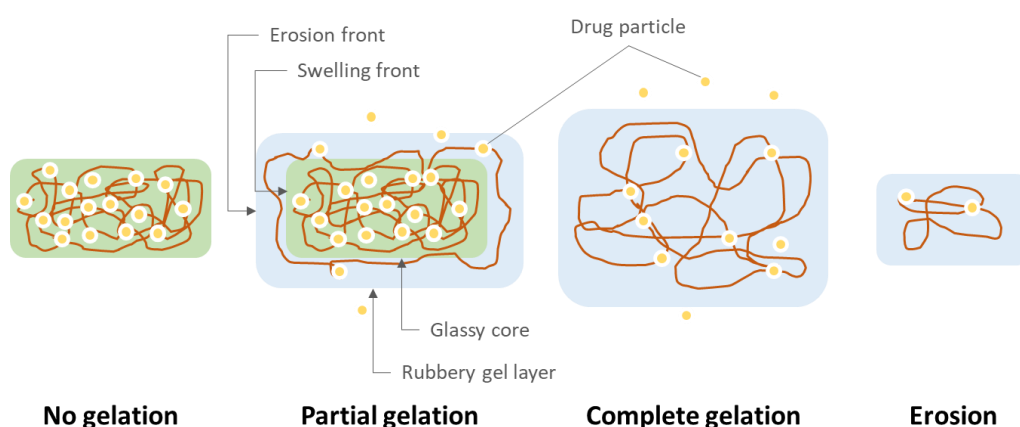


Figure 1.3. Schematic representation of drug release processes from a hydrophilic matrix, adapted from Jamzad et al. (2005)²⁹ and Aulton and Taylor (2013)³⁰.

There are two principal mechanisms controlling the release of drugs from the matrix tablets: (1) drug diffusion through the gel layer and (2) movement of drug

molecules through the erosion of the gel layer.^{27, 31-32} The solubility of APIs is an underlying factor in controlling drugs release. Generally, water-insoluble substances have limited drug diffusion from the matrix but they are released predominantly via a polymer erosion process.

1.2.5 Critical factors impacting drug release from HPMC matrices

There are two main factors generally involved with drug release from tablet formulations, formulation factors and manufacturing processes. Comprehension of these factors is essential for the optimal development of formulations. In this section, formulation factors, which include drug and excipient factors along with manufacturing processes are briefly shown. However, as HPMC factors are the main focus of the study, information about the HPMC factors influencing the release of drug from hydrophilic matrices is detailed in Section 1.2.5.2.

1.2.5.1 Drug factors

The physicochemical and biopharmaceutical properties of drug substances are considered as critical factors determining the release profile of drug from tablets, including hydrophilic tablets. The drug properties which is related to the dissolution of tablets involve pKa, particle size, surface area, solubility, drug class in the biopharmaceutical classification system (BCS)³³, polymorphism/crystallinity, hydrates and salt form.³⁴⁻³⁸ Decreasing particle size of drugs increases a dissolution rate because the specific surface area increases.³⁶⁻³⁷

In HPMC matrices, the drug particle size was the factor responsible for the changes in dissolution rates, especially in the case of insoluble drugs. For soluble drugs, the influence of drug particle size was only observable at low content of HPMC. The reduced particle size of the drug may mean that it dissolves more readily when dissolution media penetrates through the matrices resulting in a more significant role for diffusion, whereas, the larger particle sizes would be less freely dissolve, thereby behaving more prone to erosion at the matrix surface.³⁷ It is also the case for the water solubility of the drug. The release

profile of water-soluble drugs seems to be based on diffusion of drug through the gel layers, while the release of hydrophobic drugs tends to be mainly as a result of gel erosion.^{31, 39} Due to the lower solubility of drugs, the drugs are hardly dissolved and remain the solid particles showing more varied dissolution rates. The molecular weight of drug was suspected to relate to the diffusivity of drugs through the gel layer of the hydrophilic matrices, resulting in the difference in the mean dissolution times (MDT): Drugs with a lower molecular weight may have higher diffusional contribution demonstrating the faster dissolution.⁴⁰ It was found that the apparent diffusion coefficient for tetracycline hydrochloride was lower than propranolol hydrochloride. The reason for this is probably due to the fact that the molecular weight of tetracycline hydrochloride is almost double that of propranolol hydrochloride.⁴¹ A study in 2007 showed that the aqueous solubility and the size of the drug were the most important factors over other drug properties in determining drug release from HPMC 2208 (K4M) and HPMC 2910 (E4M) matrices.⁴² In addition to drug solubility, the level of drugs loaded into the matrices was also important, especially in the case that the content of the drug is high and water solubility is fair. Mitchell *et al.* found that increasing drug loading dose despite constant HPMC 2208 (K15M) level reduced the apparent diffusion coefficient of tetracycline hydrochloride.⁴¹ The conversion from tetracycline hydrochloride to tetracycline, which is a less soluble form, may have occurred.⁴¹ This conversion probably led to precipitation in the gels and restriction of drug diffusion through the gel.⁴¹ Therefore, drug release profile is determined by both the diffusion of drugs and erosion profile of the matrix tablet.³¹ Based on the BCS class, drugs in the BCS Class I (high solubility and high permeability) are generally selected for the extended release formulation.³⁰ As the rate-limiting step is drug release from the systems, it allows the modification of the formulations. Developing the extended release dosage forms is more challenging with the drugs with low solubilities such as the members in the BCS Class II and IV.³⁰ These classes inherently show a sustained release behaviour without any modification.

1.2.5.2 HPMC factors

Influence of polymer factors on the release profile of drugs from hydrophilic matrices using HPMC has been studied widely.¹² The most critical polymer factors, include HPMC polymer level, particle size, viscosity grade and substitution type, will now be discussed.

1.2.5.2.1 Concentration

Much of the previous research on HPMC-containing tablets focuses on the effects of HPMC level on the release profiles of several drugs such as tetracycline hydrochloride, propranolol hydrochloride, indomethacin, zidovudine, acetazolamide, diclofenac sodium and chondroitin sulphate from HPMC matrix tablets.^{41, 43-48} HPMC has been used as a polymeric matrix to retard the release. Increasing the HPMC level caused the reduction of the release rate in several studies.^{41, 43, 45, 47} (Figure 1.4) The MDT values of zidovudine became substantially higher in the matrix formulations containing HPMC from 10 to 40 %.⁴⁷ Avachat and Kotwal developed a fixed-dose combination containing diclofenac sodium and chondroitin sulphate in HPMC-based controlled release tablets.⁴⁶ They demonstrated that the matrices with low levels of HPMC (15 and 20 %) may experience erosion process before swelling had completed, whereas, the release of diclofenac sodium and chondroitin sulphate can be simultaneously prolonged for up to 9 hours when 40 % of HPMC was used.⁴⁶ Drug diffusivity which is proportional to the dissolution rate reduced as the apparent diffusion coefficient decreased with increasing HPMC content.⁴¹

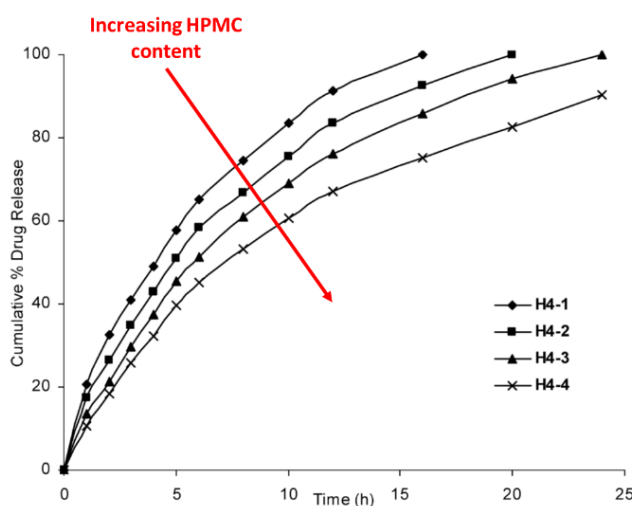


Figure 1.4. Effect of HPMC content on the release of zidovudine from HPMC matrices containing 10 (◆), 20 (■), 40 (▲) and 60 (×) %w/w HPMC 4000 cps, adapted from Ravi et al. (2008).⁴⁷

The change in release rates in relation to the amount of polymer can be explained as follows. As the polymer concentration is increased, there is more chance of the polymer to absorb water, resulting in expansion of gel thickness.⁴³ Higher HPMC level increases polymer chain entanglement⁴¹ which leads to a greater degree of swelling, tortuosity and length of drug diffusional path in the gel.^{43, 47} The gelatinous layer becomes stronger and less susceptible to drug diffusion and gel erosion. Therefore, the polymer level or the drug-to-polymer ratio is a major factor for controlling the release of drug in the matrix tablets.

A certain critical polymer level must be achieved to ensure the complete gelatinous layer and the sustained release of drug from hydrophilic matrices. Christenson and Dale⁴⁹ recommended in their patent filed in 1962 that the preferred fraction of the HPMC in the tablet is at least a third of overall formula as illustrated in the following statement. “...we (Christenson and Dale) prefer that the tablets of the present invention contain at least one part by weight of the hydrophilic, water swellable gum for each two parts of the other components of the tablet whether they be active ingredients or conventional fillers, binders or excipients.”⁴⁹ This patent claimed that with one-third of HPMC, the prolonged action of at least three to four hours in the GI tract would be achieved.⁴⁹ The HPMC level of 30 to 40 % for performing an adequate polymer gel barrier in the

controlled release tablets is also suggested by a manufacturer of HPMC (Dow chemical company, US)¹⁶. This amount of HPMC could provide the tablet formulations with more robust and highly resistant to other formulation and process variables.¹⁶ However, it is noted that drug release is also dependent on other formulation and processing factors, such as any other excipients in the formulations and drug solubility. Other factors could impact how much the HPMC level should be used in any particular formula.

1.2.5.2.2 Particle size and shape

The research on the relationship between the particle size of HPMC and the release profiles of drugs has been highlighted by several authors.^{37, 50-53} In general, it seems that the release rate of drugs is proportional to the size of HPMC. Previously, Alderman (1984) reported inefficiency of the tablets in sustaining drug release when using coarse fractions of HPMC.⁵² This work found that the matrices using HPMC 2208 (K4M) particles with the size of about 150 μm showed immediate drug release, while the releases of drugs were more sustained when the smaller fractions of HPMC were applied.

Compared to the polymer content, it appears that the effect of HPMC particle size on drug release is less obvious. The influence of particle size is increasingly strong with lower HPMC level used. It can be exemplified by the experiments done by Campos-Aldrete and Villafuerte-Robles in 1997.⁵⁰ They also provided the explanation of how the particle size impacts the release: larger particles require a longer time for the polymer to hydrate and swell; a greater pore size would be formed in the gel barrier with increasing the HPMC size fractions.⁵⁰

The critical point of polymer particle size was characterised in the matrices containing HPMC 2208 (K15M Premium) at average size range from 34 to 309 μm by Heng *et al.* in 2001 (Figure 1.5).⁵³ Both rate and mechanisms were markedly different below and above the critical point (particle size of 113 μm). Below the mean HPMC size of 113 μm , the release rates of aspirin were less subject to the particle size of HPMC and the release profiles followed the first-order kinetics, whereas, the release rates were considerably increased in the

matrices with the polymer particle size of above 113 μm and they deviated from the first-order kinetics. Another study also observed that HPMC particle size affected the lag time significantly.³⁷ There was no lag time found in the matrices produced with the 150 to 250 μm range of HPMC, demonstrating a burst release of the drug.³⁷ It can be concluded that at the low level of HPMC, not only the variation in release rate is affected by the particle size, but also the change in release mechanism is observed.

While several authors have attempted to highlight the importance of particle size, Bonferoni *et al.* (1996) investigated not only the impact of particle size but also the shape of HPMC 2910 (E4M) particles on the release performance of drug from the matrix tablets.⁵⁴ The particle of HPMC was categorised into either fibre-like or spheroidal particles. The main finding was that tablets with more fibrous-shaped particles exhibited more delayed release than the spheroidal ones. Stronger matrices could be achieved by the interlocking of fibre-like particles, impacting swelling properties of the tablets.

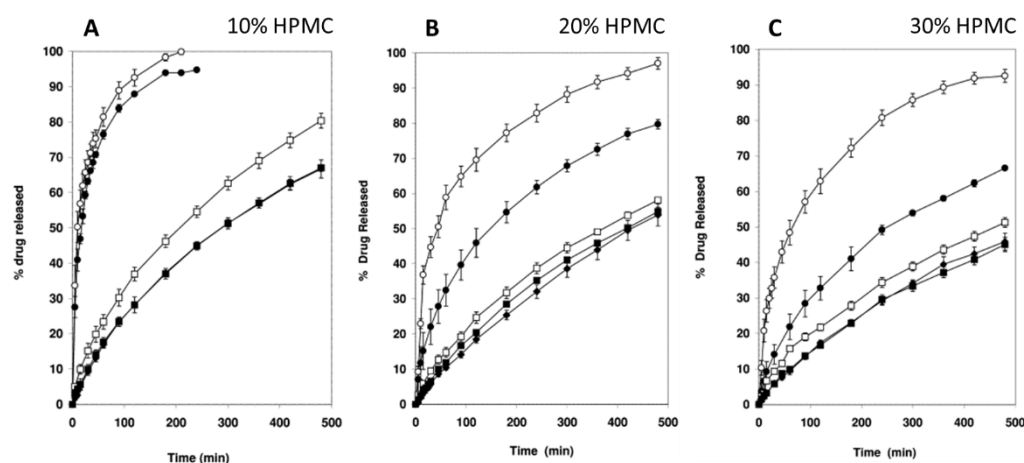


Figure 1.5. Effect of HPMC particle size on the release of aspirin from HPMC matrices containing (A) 10, (B) 20 and (C) 30 %w/w HPMC tablets with the mean particle size of 34 ($\blacklozenge$), 58 ($\blacksquare$), 113 ($\square$), 180 ($\bullet$) and 309 ($\circ$) μm , adapted from Heng *et al.* (2001).⁵³

1.2.5.2.3 Substitution types and viscosity grades

Hydration rate may vary depending upon the relative percentage of methoxy and hydroxypropoxy groups (i.e. substitution type). Details in substitution types are described in Section 1.2.3. The hydroxypropyl substituent group contains a

secondary hydroxyl on the number two carbon. Having high hydroxypropyl substituents leads to increasing the hydrophilicity of HPMC. In contrast, hydrophobic methoxyl substitution reduces hydrogen bonding within and between HPMC particles.⁵⁵ It was found that the rate of hydration in commercially available Methocel was as follows; HPMC 2208 (Methocel K) > HPMC 2910 (Methocel E) > HPMC 2906 (Methocel F).⁵⁶ The order of hydration rate is in line with the methoxy/hydroxypropyl substitutions. The time to hydrate is important, especially for the matrices containing highly water soluble APIs. If the matrix is too slow to absorb water and not quick to hydrate, there would be a propensity for dose dumping before the matrix can develop the complete gel layer.

The burst effect is influenced by the viscosity of the HPMC at fixed polymer level and substitution type.⁴⁵ A study of viscosity on the burst effect was done by Huang and co-workers in 2004 using a commercial Metolose with three viscosity grades (mPa·s), 4000, 15000, 30000. Metolose 4000 showed the lowest burst effect, compared to the other grades.⁴⁵ This can be attributed to the fact that the gel from Metolose 4000 tended to form more easily than from the higher viscosity grade. However, in the later stage, higher viscosity Metolose showed slower drug release. This result is in agreement with previous studies by Ford *et al.* (1985) that the use of the lowest viscosity grade of HPMC led to the highest release rate.⁵⁷⁻⁵⁸ The highest viscosity grade of HPMC might possess the stronger strength of the gel developed.

1.2.5.3 Non-HPMC excipients and formulation factors

There are a few studies investigating excipient parameters influencing the release of drugs.⁵⁹⁻⁶¹ Different disintegrant content influenced the extent of dissolution of ibuprofen tablets.⁵⁹ A batch in the absence of a disintegrant did not disintegrate, leading to meagre drug dissolution rates. The increased disintegrant concentration resulted in faster disintegration and improved dissolution. With respect to HPMC matrices, the release profiles may be attributed to the solubility of excipients. The insertion of insoluble excipients had a more pronounced negative effect on the release rate of the matrices with a

soluble drug than those with a lipophilic drug.⁶² Studies also found that addition of soluble diluents such as lactose or microcrystalline cellulose (MCC) to the HPMC matrices in the presence of soluble drugs resulted in faster drug release profiles compared to using less soluble excipients.^{45, 63-64} Even though the molecular weights of lactose and MCC differ, the replacement of lactose with MCC and *vice versa* did not significantly modify the release.⁴³ An explanation for these findings is that drug release is determined by diffusion in the case of hydrophilic drugs. Since the drug diffusion coefficients are greatly dependent on the water content of the system, the more soluble excipients would be more susceptible to water penetration, thus increasing the mobility of water and its amount in the gel. It was found that using a low level of the lubricant (less than 2 % magnesium stearate) did not significantly influence the release rate.⁴⁵

1.2.5.4 Manufacturing factors

There are a number of processing parameters found as critical manufacturing attributes impacting dissolution characteristics of tablets such as lubricant blending time, compression force, order of mixing, drying and coating method, granulation-dependent factors and equipment capability.³⁴ Generally variation in compaction pressure may alter the tortuosity or porosity of tablets, resulting in the alteration of release profiles.⁶⁵ However, compaction pressure and compression force seem to show minimal or no impact on drug release of HPMC matrices.^{37, 51, 57} A study by Huang *et al.* did not observe a noticeable change in the dissolution profiles when changing the compaction pressure from 100 to 200 MPa.⁴⁵ Another study reported a negligible effect on drug release from HPMC matrices with different compression forces (3 to 12 kN) even if compression force is a critical factor impacting tablet hardness.³⁷ In term of the manufacturing method, it was found that the drug release profile of tablets with direct compression method was fastest, followed by the dry granulation and wet granulation processes.⁶⁶ It may be attributed to variation in the porosity of tablets due to a different method.

1.2.5.5 Other factors

The dissolution conditions such as dissolution apparatus, agitation rate, medium pH, media composition, buffer condition and temperature affect dissolution.^{35, 44, 67-70} A study by Hamed *et al.* regarding the solubility of carvedilol found that the dissolution profile behaviour of carvedilol was relevant to its solubility.⁶⁹ Its release was almost complete in acidic dissolution media and relatively low in a simulated intestinal fluid as carvedilol is a weakly basic drug. A study of Kavanagh and Corrigan in 2004 discussed the influence of agitation rate on the swelling and erosion properties of HPMC.⁶⁸ It was found that there was an inverse relationship between the extent of swelling and agitation rate but a direct relationship between the erosion rate and agitation rate. The development of an appropriate dissolution method is required on a case-by-case basis. Therefore, it is essential to first choose an established compendial dissolution condition for the purpose of drug release determination. Otherwise, the development of the optimal controlled method is needed.

Apart from the dissolution conditions, it seems that the release rate of drugs is related to the morphology of tablets such as shape and size.⁷⁰⁻⁷¹ The difference in tablet shape or size may alter the relative surface area/volume of dissolution media, thus impacting water penetration. Reynold *et al.* (2002) demonstrated that the release of soluble drugs from HPMC matrix tablets with similar ratios of surface area/volume was similar within the same tablet shape or even among different shapes.⁷² Changing the relative surface area/volume, therefore, can also be done to optimise the release of hydrophilic matrix tablets.

1.2.6 Percolation theory in understanding drug release

Percolation theory is a field of multidisciplinary research involving a phase transition of a component in a system concerning geometric properties. Percolation theory studies the formation of clusters (the groups of neighbouring occupied sites in the system).⁷³ The ‘percolation threshold’ is the critical point at which a component of the lattices starts to connect and form an infinite or a percolating cluster that extends from one side of the system to the other. The

possibility of percolation depends on the volume fraction of each component instead of its weight.⁷³ Below this point, there was no such infinite cluster. The structure of the system is expectedly different between below and above the percolation point, thereby exhibiting a significant change in the properties.⁷⁴⁻⁷⁵

The concept of percolation theory has been widely applied in various fields of research, especially polymer sciences.⁷⁶⁻⁸⁰ For a matrix tablet point of view, a percolating cluster can be theoretically defined as a group of neighbouring particles of the same component which produces a continuous phase through the matrix.⁷⁵ The application of percolation theory for pharmaceutical research initially appeared in the study led by Leuenberger in 1987.⁸¹ The study found that remarkable changes in the properties of binary mixtures, such as disintegrating activity and intrinsic dissolution rate, linked to the percolation threshold of active substances and excipients in the systems.⁸¹

Several studies investigated the percolation threshold of components in hydrophobic⁸²⁻⁹⁰ and hydrophilic matrices⁹¹⁻⁹⁴ and evaluated the effect of percolation on drug release kinetics with regards to polymer and drug concentrations. In the case of inert matrices which contains insoluble materials on the outer phase for controlling the release of the active substance, the percolation threshold of the drug was tremendously important for drug release.⁸⁵ The matrices needed to contain the drug at above its percolation threshold; otherwise the drug would exist in the inert matrix systems instead of being released. On the other hand, the critical threshold corresponds to a hydrophilic polymer instead of the drug in the case of hydrophilic matrices. The percolation threshold of the matrix-forming polymer in hydrophilic matrices seemed to be significant in both binary⁹¹ and multicomponent systems^{92, 94}. For example, it was found that an infinite cluster of HPMC formed while there was a sudden change in the values of kinetic parameters.⁹¹⁻⁹² Using the content of HPMC higher than its percolation threshold confirmed the uniform hydration and extended-release of verapamil HCl.⁹² However, the matrix with the amount of the polymer below the critical threshold would not have an efficient gel strength, thus behaving as an immediate release system.⁹⁵

The effect of percolation in relation to polymer concentration has been discussed. Apart from that, this concept was used to provide a reason why the particle size of tablet components governed release kinetics. A linear relationship between the particle size of drugs and percolation threshold was evident in inert matrices.⁸³ Another study investigated percolation in swellable matrices and found that components with smaller particle size show relatively lower percolation thresholds demonstrating higher efficiency to percolate.⁹² However, it is worth noting that the drug and excipient particles might deform during the compacting process. Therefore, it may not be easy to speculate about the same relationship in all cases. The concept of percolation helps the scientist optimise formulation design, especially in the matrix systems, in order to achieve effective controlled release systems.

1.3 Drug release study of HPMC matrices

1.3.1 Dissolution testing

Requirements from regulatory bodies are complied with tablet formulations to assure their performance. There are general tests for tablets such as dissolution, disintegration, friability and uniformity of dosage units. The dissolution of a tablet is a process that a drug substance releases from the tablet. It involves two main stages, disintegration and API dissolution. Disintegration occurs when the tablet has a contact with an aqueous fluid resulting in the tablet becoming smaller fragments, which may be coarse or fine particles. Then the API which is liberated from the tablet dissolves. The dissolution of the API is controlled by numerous factors, e.g. the solubility and physicochemical properties of APIs in the medium.

Usually, measurement of drug release from oral dosage forms is quantified using *in vitro* dissolution testing. The ultimate goal of *in vitro* dissolution tests is to ensure continuous product quality and allow a waiver of conducting *in vivo* bioequivalence studies. Information obtained by *in vitro* testing can describe drug release characteristics of products in a number of stages. In the initial stage of drug development, *in vitro* dissolution testing becomes an evaluation tool in formulation design and optimisation and also forecasts potential issues with *in vivo* bioavailability. A good dissolution method with adequate discriminating power can detect formulation changes that affect the dissolution rate of a drug product. Dissolution testing is required by regulatory authorities for marketing authorisation. After drug product approval, dissolution testing is routinely performed in quality control to demonstrate batch-to-batch consistency before the release of each batch to the market. Dissolution documentation may be used to provide evidence to support the justification of scale-up and post-approval changes such as minor changes in formulation or manufacturing process or changes in packaging materials. Due to the aforementioned reasons, *in vitro* dissolution testing is a ‘gold standard’ for quality assessment of pharmaceutical dosage forms.

Compendial dissolution methods for solid oral dosage forms described in the USP¹⁵ and British⁹⁶ Pharmacopoeias include four types of apparatuses, the basket, the paddle, the reciprocating cylinder and the flow-through cell. Apparatus II (the paddle) is relatively used as an apparatus of choice for several formulations, compared to Apparatus I (the basket). Apparatus III (the reciprocating cylinder) is in use in the study of pH profiling. Apparatus IV (the flow-through cell) is usually for the formulations containing poorly soluble APIs. Typically, the procedures for Apparatus II are listed as follows⁹⁶; the dissolution medium is equilibrated to 37 ± 0.5 °C before the dissolution test. The dosage unit is transferred to the apparatus for the dissolution test at the specified rotation speed rate in each monograph. At each of the times stated in the monograph, a specimen from a zone midway between the surface of the dissolution medium and the top of the rotating blade is withdrawn. The analysis using a suitable assay method described is then performed. UV-visible spectroscopy (UV-vis) and high performance liquid chromatography (HPLC) are generally used for the quantitative determination of drugs. UV-vis is relatively economical and less time-consuming. However, placebo interference caused by the overlap of the absorption bands may be found in several cases. HPLC may be preferable as it is rather appropriate for separating mixtures of materials.

1.3.2 Limitation of dissolution testing

As mentioned in Section 1.3.1, the nature of *in vitro* dissolution testing for solid oral dosage forms requires sample collection in the form of solutions. As a result, a direct measurement from the solid form of tablets is intrinsically not possible. The chemical information is not spatially resolved so that the distribution of components in the systems is unknown. In addition, complex information such as the release mechanism of drugs from the formulations is not obtainable. Compendial dissolution apparatuses cannot mimic the dynamic nature of dissolution occurred in the human's GI tract. Until now, the extensive research on HPMC has particularly highlighted factors influencing on the release of drugs from the hydrophilic matrices (Section 1.2.5.2). However, dissolution testing

cannot provide supporting evidence for the explanation of this existing link. Even though dissolution testing is a standard method in the analysis of solid oral dosage forms but it only provides a rudimental picture of the performance of the pharmaceutical products.

1.3.3 Use of imaging techniques in studying drug release of hydrophilic matrices

Early studies of hydrophilic matrices applied the use of several imaging techniques for the study of gel growth in the hydrogel-based systems e.g. optical method, confocal laser scanning microscopy (CLSM), nuclear magnetic resonance (NMR) and magnetic resonance imaging (MRI), *etc.* Recently, vibrational spectroscopy techniques, such as Fourier-transform infrared spectroscopy (FTIR) and near-infrared spectroscopy (NIR) have been applied to hydrophilic matrices in an attempt to track the localisation of drugs in the matrix systems. Each technique can provide valuable information for formulation development as well as a number of limitations and challenges. This section gives an overview of imaging techniques alongside their applications focused on HPMC matrices. Their potentials are highlighted to gain a better scientific understanding of the release mechanism of drugs from hydrophilic matrix tablets.

1.3.3.1 Optical imaging method

The transport phenomena of HPMC-based matrix tablets in water (i.e. the water uptake, gel swelling and polymer erosion) were investigated using optical imaging. Optical imaging in conjunction with the use of colourants or water-soluble coloured drugs was demonstrated in a number of studies.^{27, 97-98} Two sharp fronts of the gel layer in the matrix systems were generally identified, the ‘erosion front’ and ‘swelling front’. The erosion front separates the matrix and the aqueous medium, whereas, the swelling front is where there is a transition between the glassy state and rubbery state. In 1996, Colombo and his group found the third boundary inside the gel layer by the observation of the highly-loaded coloured water-soluble drug, buflomedil pyridoxal phosphate (BPP), in

HPMC matrices.²⁷ This new front separates the layer between undissolved and dissolved drugs. It was termed ‘undissolved drug front’ or ‘diffusion front’.⁹⁹ The distance between the diffusion and erosion front was found to be crucial for release kinetics.²⁷ It was found that the visibility of the diffusion front was associated with drug loading as high drug loading increased to the presence of an undissolved drug layer (Figure 1.6).¹⁰⁰ Studies found that the dimensional change and the front movement of the matrix during hydration were different with varying drug solubility.^{97, 101} In the case of water-soluble drugs, hydration of the matrix caused the dissolution of drugs upon the contact of the tablet with water. Dissolved drugs left pores which could ease penetration of water. Polymer relaxation also gradually occurred resulting in the expansion of the polymer chains and hydration of adjacent drugs. The diffusion front then moved inwards in the matrix and demonstrated the concentration gradient of the dissolved drug. When there was the complete change of the polymer from the glassy to rubbery state, all drug particles are already dissolved. In the case of water-insoluble drugs, the drugs stayed in the dry core until the phase transition of the polymer occurred. Relaxation of polymer chains led to ‘drug particle translocation’. The diffusion front then came outwards and converged with the erosion front. The existence of undissolved particles in the gel reduced the entanglement of polymer chains which decreased the gel thickness and the resistance of gel towards erosion.^{97, 101}

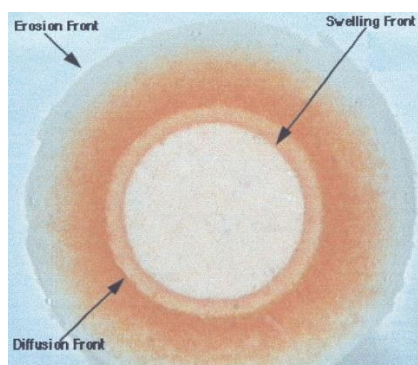


Figure 1.6. Observation of three fronts using optical imaging and colourimetric techniques by Colombo et al. (1999)¹⁰⁰. The matrix contains 60% BPP (w/w) taken after 240 min of swelling. In the dry state, the colour of the drug was light yellow, whereas, in solution, it ranged from yellow to orange as the concentration increased.

The optical method, in conjunction with colourimetric analysis, can describe the phenomenon during tablet hydration and gel layer formation. It enables an *in situ* time-series experiment. However, a significant drawback of this technique is that it cannot be applied to identify the location of non-coloured drugs so that this technique is not chemically specific and not suitable for all drugs.

1.3.3.2 CLSM

CLSM is a form of fluorescence microscopy that combines high-resolution optical imaging with depth selectivity. Hence, it allows three-dimensional (3D) reconstructions of thin sections with versatile fluorescence measurements in the systems containing fluorophore molecules.¹⁰² A group led by Melia extensively researched into gel layer formation, especially in hydrophilic matrices.^{61, 103-104} CLSM allows the study of the temporal development of the gel layer in the early hydration stage.¹⁰⁴ However, one main challenging issue is that the level of fluorescence needs to be suitable to justify the boundaries in the gels. Mason *et al.* (2015) utilised CLSM for identifying the percolation threshold of HPMC in the tablets.⁶¹ It was found that matrices with polymer contents below the threshold resulted in irregular gel layer formation and failure of early gel layer development.⁶¹ The main limitation of this technique is that only pre-chosen markers can be detectable demonstrating that it offers low chemical specificity.

1.3.3.3 NMR spectroscopy and MRI

The principle of NMR spectroscopy and MRI is about the magnetic properties of atomic nuclei. The sample with nuclei is applied with the strong homogeneous magnetic field and radiofrequency electromagnetic radiation. These nuclei tend to align with the direction of the magnetic field and create a net magnetic moment. NMR imaging or MRI is formed from the NMR signal generated by the nuclei (e.g. ^1H , ^{19}F , ^{14}N , ^{15}N , ^{13}C , ^{31}P , *etc.*). MRI is a non-invasive tomographic imaging technique enabling the internal characteristics of objects and events on macroscopic and microscopic scales in the forms of two-dimensional sectional images and 3D volumetric images.

In the study of matrix tablets, the effect of matrix types on the static and dynamic processes during gelation (e.g. hydration, swelling and erosion) was compared.¹⁰⁵⁻¹⁰⁶ The study of HPMC-based matrix tablets is the vast majority amongst other matrix systems in this field.¹⁰⁷ The potential applications of MRI in the HPMC matrices are generally for the identification of fronts or transition zones as well as the spatial and temporal studies of gel development.¹⁰⁷ Rajabi-Siahboomi *et al.* (1994) initially studied the developing surface hydrated layer from different substitution types of HPMC matrices (Methocel E4M, F4M and K4M) and they found that HPMC 2208 (K4M) formed the best diffusion barrier amongst others.¹⁰⁸

A major drawback of MRI is that MRI provides low chemical specificity. Commonly, ¹H NMR spectroscopy is used to analyse water behaviour in the gel. However, the solid tablet matrix is not directly observed using MRI. Most drugs cannot be identified. However, previous studies carried out ¹⁹F NMR imaging experiments to study the release of drugs containing fluorine atoms, e.g. triflupromazine HCl¹⁰⁹, 5-fluorouracil¹⁰⁹ and fluvastatin sodium¹¹⁰⁻¹¹¹. As a non-spectroscopic technique, the quantification of drugs is also an issue in NMR.

1.3.3.4 Vibrational spectroscopic imaging

Vibrational spectroscopy such as FTIR¹¹² and NIR¹¹³⁻¹¹⁴ have recently been applied to the study of HPMC behaviour in hydrophilic matrices. They allow both quantitative and qualitative analysis. Chemical imaging obtained from vibrational spectroscopy contains the spectral data of chemical bonds in molecules and their spatial information. Vibrational spectroscopy comprises of four different techniques which characterise molecular vibrations at different wavenumber regions, including terahertz or far-infrared; FIR (400 - 10 cm⁻¹), FTIR (4000 - 400 cm⁻¹), NIR (13,000 - 4000 cm⁻¹) and Raman spectroscopy (4000 - 400 cm⁻¹). FIR measures intermolecular vibrations, enabling the identification of crystalline APIs. However, amorphous APIs and most excipients are transparent or semi-transparent in the region of FIR. FIR then is not widely used in the analysis of pharmaceutical products.

FTIR spectroscopic imaging

Mostly, FTIR measures the absorption of the infrared light by a sample. It detects fundamental molecular and intramolecular vibrations. A range of sampling configurations can be employed in the analysis using FTIR, e.g. transmission, attenuated total reflection (ATR) and reflection. The transmission mode requires a thin sample for the IR light to pass through so that the tablet sample is not suitable for the transmission mode. ATR is in widespread use as it needs no sample preparation and sample thickness is not an issue. ATR-FTIR was successfully implemented with USP dissolution tests to monitor a simultaneous process during dissolution in a few studies.^{26, 115-116} Additionally, FTIR could quantify the content of compounds in the surface layer adjacent to the ATR crystal. It was possible to observe the chemical state of the drug which depended on the change in microenvironmental pH due to pH modifiers.¹¹⁷⁻¹¹⁸ FTIR imaging provided salt formation data which greatly affected the dissolution of the drugs.¹¹⁸ The lateral resolution of FTIR imaging is limited by light diffraction and the optical capability of each system (i.e. the numerical aperture of an objective and the signal-to-noise ratio of the system).¹¹⁹ The estimated spatial resolution of the FTIR was reported to be 15 – 20 μm in FTIR with a diamond ATR element.¹²⁰

NIR spectroscopic imaging

NIR spectroscopy collects the excitation of first and second overtones and combination bands which require high energy (short wavelength) in the NIR range. With the diffuse reflectance mode, no sample preparation is required. A few studies illustrated the use of NIR imaging for the study of HPMC matrices in order to observe the gel layer development *in situ*.¹¹³⁻¹¹⁴

Raman mapping

Raman spectroscopy is a spectroscopic method based on the scattering of electromagnetic radiation. When the light from the source of monochromatic radiation (laser in modern technology)¹²¹ interacts with the molecules, most scattered energy from the molecules has the same frequency as the incident

radiation. It is termed 'Rayleigh scattering'. However, some of the photons (1 in 10^6 photons)¹²² scatter inelastically. 'Inelastic scattering' or the 'Raman effect', which was named after the first discovery by Sir C.V. Raman in 1928¹²³, can occur in two possible ways. The first one is 'Stokes scattering' in which the Stokes photons have less energy than those of the exciting radiation. Another is 'Anti-Stokes scattering' which is the re-emission of the higher energy photons than the exciting energy result.^{121, 124} Raman data is complementary to FTIR data as they record molecular vibrations in the same spectral region.

Although Raman spectroscopy has never been used with HPMC matrices, it has featured prominently in the release studies of other pharmaceutical tablet formulations over the last decade.¹²⁵⁻¹²⁹ Raman mapping facilitates the determination of API distribution. Raman spectroscopy highlighted the difference of drug movement in three-layered tablets which was in response to the characteristics of the polymer.¹²⁸ As the Raman scattering process is intrinsically weak and is easily interfered by fluorescence, it is likely that Raman spectroscopy has difficulties in obtaining information linked to a low dose of analytes.¹³⁰ However, a number of studies have used Raman spectroscopy for detecting impurities or other minor components such as lubricants in pharmaceutical tablets.¹³¹⁻¹³² Raman microscopy and advanced multivariate data analysis method were used to identify magnesium stearate at a concentration as low as 0.2 %w/w.¹³¹ The applications of Raman spectroscopy were summarised in the literature such as the assessment of sample homogeneity and particle size distributions, the determination of polymorphism and structural form changes and the detection of counterfeit drugs.¹³³

1.3.3.5 Mass spectrometry imaging

Secondary ion mass spectrometry (SIMS) involves the bombardment of primary ions under ultra-high vacuum conditions. As a result, it causes a collisional cascade in the uppermost layer of a solid surface leading to sputtering of ionised particles. Sputtered (emitted) secondary ions are subsequently extracted and directed into a mass spectrometer. The flight time of the secondary ions is dependent upon their mass. These emitted species accurately represent the

surface-sensitive chemistry of the sample. There are well-established techniques predominantly used in the field of SIMS including Nano-SIMS, matrix-assisted laser desorption/ionisation (MALDI) and time-of-flight secondary ion mass spectrometry (ToF-SIMS). Nano-SIMS offers a nanoscopic scale resolution but it is limited to elemental analysis. Only elements and small fragments are collected in the Nano-SIMS systems¹³⁴. While large biomolecules may not be detectable using Nano-SIMS, MALDI is capable of collecting larger molecules such as proteins or peptides. However, the disadvantage of MALDI is that it is not possible to detect elements and molecules in the low mass range (below a mass to charge ratio (m/z) of 500)¹³⁴. Among these techniques, ToF-SIMS is an in-between as it can achieve a lateral resolution in a submicron scale with a good ion detection range between about 1500 Da and 2500 Da¹³⁴. ToF-SIMS provides elemental and molecular chemical information of a solid surface, allows monolayer imaging, renders 3D reconstruction and enables compositional analysis through a material as a function of depth.

ToF-SIMS is broadly applied in the characterisation of inorganic compounds.¹³⁵⁻¹³⁸ As well, there is increasing recognition of ToF-SIMS applications in the analysis of the surface and depth profiling of numerous organic systems.¹³⁹⁻¹⁴¹. However, only a few studies have performed this technique for the analysis of pharmaceutical systems until now¹⁴²⁻¹⁴⁴. ToF-SIMS, to the best of our knowledge, has not yet been exploited in the field of hydrophilic matrices. However, ToF-SIMS provides several advantages, including elemental and molecular surface-sensitive characterisations, high spatial resolution (down to 200 nm)¹⁴⁵, high molecular specificity, high mass resolution and the ability to develop 3D imaging. Compared to spectroscopic imaging, ToF-SIMS is better in spatial and depth resolutions as the resolution of ToF-SIMS is not limited by the diffraction limit. It would then potentially and alternatively be a chemical imaging technique of choice for the characterisation of hydrophilic matrices. There are generally three functions that ToF-SIMS can be used: (1) surface spectroscopy or static SIMS, (2) surface chemical imaging and (3) depth profiling and 3D imaging or dynamic SIMS.

Work by Belu *et al.*¹⁴³ is a good illustration of small molecule API and excipient analysis in controlled release drug delivery systems. Theophylline, paracetamol, and prednisolone were molecularly identified in the positive ion polarity mode of ToF-SIMS. All chemicals showed parent intact molecules and fragment ions that were helpful for being a fingerprint for each drug. Secondary ion peaks at m/z 181, 152, 589 were used to characterise theophylline, paracetamol, and prednisolone, respectively. By way of excipient identifications, they also presented the positive ToF-SIMS spectra of lactose, amylose, microcrystalline cellulose (Avicel PH101), glycerol monostearate, barium sulphate, and ethylcellulose dispersion (Surelease).¹⁴³ Another study performed ToF-SIMS for identifications of foreign matters. ToF-SIMS increased the sensitivity resulting in being able to detect a very low concentration of piroxicam (m/z 332) as a contaminant of theophylline tablets.¹⁴⁶

Visualising the spatial distribution of drug and excipients in pharmaceutical systems is the major capability of ToF-SIMS. Scoutaris *et al.* inkjet-printed different antihypertensive drug/polymer formulations as micro-dot arrays.¹⁴⁷ ToF-SIMS coupled with multivariate curve resolution image analysis was applied to evaluate the uniformity of the resulting spots.¹⁴⁷ ToF-SIMS was utilised for detecting and mapping different crystalline states of APIs.¹⁴⁸ This application is useful in the observation of the crystalline - amorphous transformation leading to undesirable effects in pharmaceutical systems. The chemical distribution of excipients on a tablet's surface was demonstrated in a few studies.^{142, 149} Davies *et al.* suggested a patchwise lubricant film distribution rather than a continuous monolayer film.¹⁴⁹ This view was consistent with the work by Hussain and colleagues that illustrated the blotchy pattern layer of 0.5 %w/w magnesium stearate on sodium chloride tablets using SIMS.¹⁴²

The capability to obtain data about the chemistry of compositions below the surface is possible to achieve by dynamic-SIMS. This application exposes the underlying material by etching the surface layer of a sample to develop the depth profiles and 3D images. Bailey *et al.* analysed a series of polymer multilayer structures composed of polystyrene (PS) and polyvinylpyrrolidone (PVP).¹⁵⁰ They were able to render the 3D images of chemical composition with the

calculated thickness between each layer of a PS and PVP multilayer system and demonstrate depth profiling. The 3D positive ion ToF-SIMS image (Figure 1.7) illustrates the homogeneity or heterogeneity of the chemical composition and reveals an imperfection detected in a PS layer. The compositional effect in a binary system of codeine and poly(lactide), which is related to secondary ion yield, was observed by Shard *et al.*¹⁵¹ This work reported that there was an increasing trend in the intensity of one component, contrarily, a decreasing trend of one another. ToF-SIMS was used to characterise the depletion of the drug from stents.¹⁵²⁻¹⁵⁴ Despite its useful capability, there have been no studies performing ToF-SIMS depth profiling for tablet analysis so far. The attractive area of label-free imaging of metabolites in animal tissues and organs can be found in the literature.¹⁵⁵

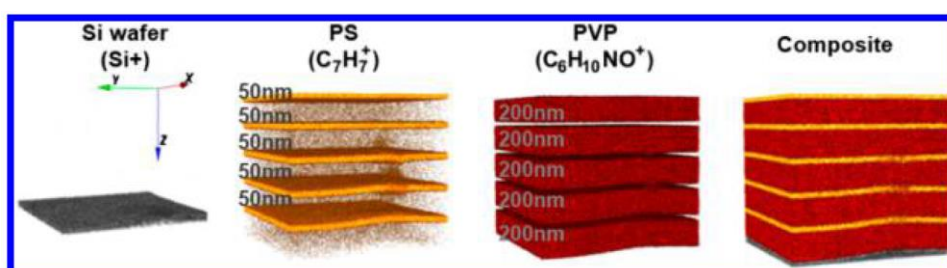


Figure 1.7. Reconstructed 3D positive ion imaging of polymer multilayer structures ($200 \times 200 \mu\text{m}$ area), adapted from Bailey *et al* (2015).¹⁵⁰

Table 1.3. Summary of imaging techniques applied for the release studies of HPMC matrices.

Technique	Advantages	Limitations	Factors impacting release
Optical photography	- Economical - Temporal resolution	- Low chemical specificity	Drug loading ^{100, 156} , drug solubility ^{97, 100-101} , HPMC concentration ¹⁵⁷ , HPMC viscosity ^{156, 158} , Paddle rotation speed ²⁷ and tablet porosity ²⁷
CLSM	- Higher-resolution imaging, compared to optical method - Non-invasive - 3D reconstructions	- Low chemical specificity (non-fluorophore systems)	HPMC concentration ⁶¹ and non-HPMC excipients ¹⁰⁴
NMR imaging / MRI	- Non-invasive - 3D technique	- Low chemical specificity (non-fluorine systems) - Limited quantification	Molecular weight of drugs ¹⁰⁹ , HPMC substitution types ¹⁰⁸ , non-HPMC excipients ¹⁵⁹⁻¹⁶⁰ and dissolution conditions ^{110, 161}
Vibrational spectroscopic imaging	- Chemical specificity - Quantitative	- Lower surface sensitivity, compared to ToF-SIMS - The spatial resolution of several microns, limited by the diffraction limit - Non-elemental specific	Non-HPMC excipients ¹¹⁷⁻¹¹⁸ , HPMC concentration ¹¹³ , drug loading ¹¹⁴

1.4 Injectable hydrogels in the use of cartilage regeneration

1.4.1 Cartilage damage

Arthritis is a simple term which refers to symptoms relating to joint pains and joint disease. In 2017, almost 25 % of adults were doctor-diagnosed with arthritis.¹⁶² By conservative estimates between 2010 - 2012, nearly 50 % of elderly people were diagnosed with arthritis.¹⁶³ Osteoarthritis (OA), which is the most common form of arthritis, is a group of chronic conditions involving the progressive deterioration of the cartilage in joints and associated underlying bony alterations. It can affect any joints, predominantly in knees and hips. The likelihood of developing OA is mainly related to ageing. In addition, it links to multifactor, including gender, ethnicity, genetics, hormonal status, bone density, nutritional factors, obesity, acute joint injury, occupational factors and sports.¹⁶⁴ The net reduction in the amount of cartilage matrix which is caused by excessive degradation and/or deficient synthesis may lead to several joint symptoms such as pain, stiffness, limited range of motion and joint deformity.

The symptoms of OA can be relieved by pharmacological therapies including oral analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, naproxen, *etc.* However, pharmacological treatment can only control pain and inflammation. Specifically, the causes of joint degeneration are not treated with pain medications. It has been found that the prolonged use of NSAIDs can trigger GI side effects¹⁶⁵⁻¹⁶⁶ and increase the risk of having heart failure¹⁶⁷⁻¹⁶⁸. The surgical treatment of OA such as osteotomy, arthroscopy, arthrodesis, and arthroplasty can be considered. However, surgical procedures are invasive and healing time might be long.

1.4.2 Preparation of hydrogels

To regenerate damaged cartilage by injecting *in situ* forming hydrogels at the specific site is one of the potentially promising strategies for patients who develop OA. These therapeutic forms are easy to administer and less invasive

compared to surgical operations. *In situ* forming hydrogels can serve as a three-dimensional temporarily support matrix aiming to restore and maintain cartilage functions. The major component of hydrogels is water which mimics the natural extracellular matrix (ECM). The porous framework of hydrogels allows sufficient transportation of nutrients and waste products. This approach can also be utilised in a wide range of patients including ones with irregular defects.

Enzymatic crosslinking to generate *in situ* forming hydrogels is a method that can be applied at physiological conditions. The oxidative coupling reaction of tyramine residues from polysaccharide-tyramine conjugates can be catalysed by horseradish peroxidase (HRP) enzyme and in the presence of hydrogen peroxide (H_2O_2). HRP reacts with oxygen from the peroxide leading to a change in the oxidation state of the HRP heme group. In a series of steps, free radicals are formed on the aromatic ring or phenolic group of the tyramines enabling C-C and C-O coupling. The schematic reaction is shown in Figure 1.8. Previous research showed that polymer concentration¹⁶⁹⁻¹⁷¹, the degree of substitution of tyramine residues¹⁶⁹⁻¹⁷⁰ and amounts of HRP¹⁷⁰ and H_2O_2 ¹⁷⁰ affects the crosslinking density and thereby the final properties of these *in situ* forming hydrogels.

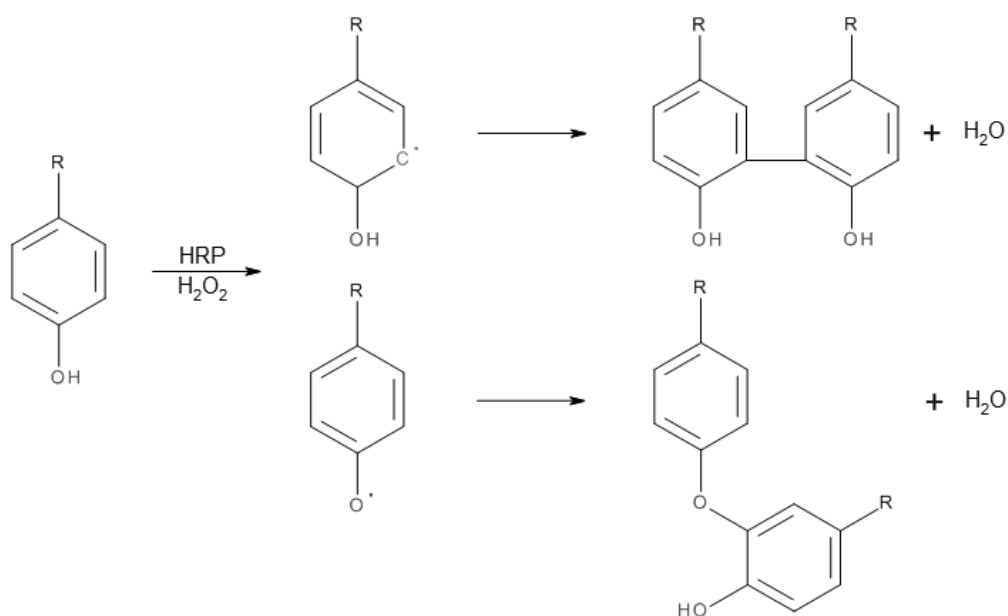


Figure 1.8. Schematic representation of enzymatic oxidative crosslinking of tyramine residues with HRP and H_2O_2 .

Another benefit of injectable hydrogels is the ability to serve as a carrier system for direct loading of cargos such as bioactive molecules, cells, peptides or proteins. Several studies have reported the potentials of growth factor delivery in tissue engineering.¹⁷²⁻¹⁷⁵

1.4.3 Platelet lysate

Human platelet lysate (PL) is a source of a tremendous number of growth factors and cytokines. It is a turbid, light-yellow liquid obtained from human blood platelet concentrates. There are several processes used to induce platelet concentrates, useful for the release of growth factors and other bioactive molecules, involving freeze/thaw-mediated platelet lysis, sonication or chemical treatment.¹⁷⁶ PL can be applied in various fields of regenerative therapies. Recent studies in cartilage regeneration, in particular, suggested that PL is possibly linked to the cell proliferation of chondrocytes¹⁷⁷ and joint-resident mesenchymal stem cells (MSCs)¹⁷⁸ leading to chondrogenesis and cartilage formation. As PL is a source of autologous therapeutic molecules, the utilisation of human PL in regenerative medicines emerges the concept of self-healing strategies to avoid the risk of xenogeneic immune reactions. PL normally contains 55-65 mg/ml of total proteins including albumin, immunoglobulin G, fibrinogen, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor isoforms (PDGF-AA, -AB and -BB), transforming growth factor- β (TGF- β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and basic fibroblast growth factor (b-FGF).^{176, 179} The typical protein composition and content in human PL is depicted in Figure 1.9. In tissue engineering, a previous study successfully integrated PL into polysaccharide-based hydrogels to develop a potential approach for cartilage repair.¹⁸⁰ This study confirmed the effectiveness of PL supplementation in hydrogels including chemoattractant properties and source of anabolic growth factors.¹⁸⁰ Recent study found the potential of injectable platelet lysate-hyaluronic acid-tyramine hydrogel as a cell delivery system for cartilage regeneration.

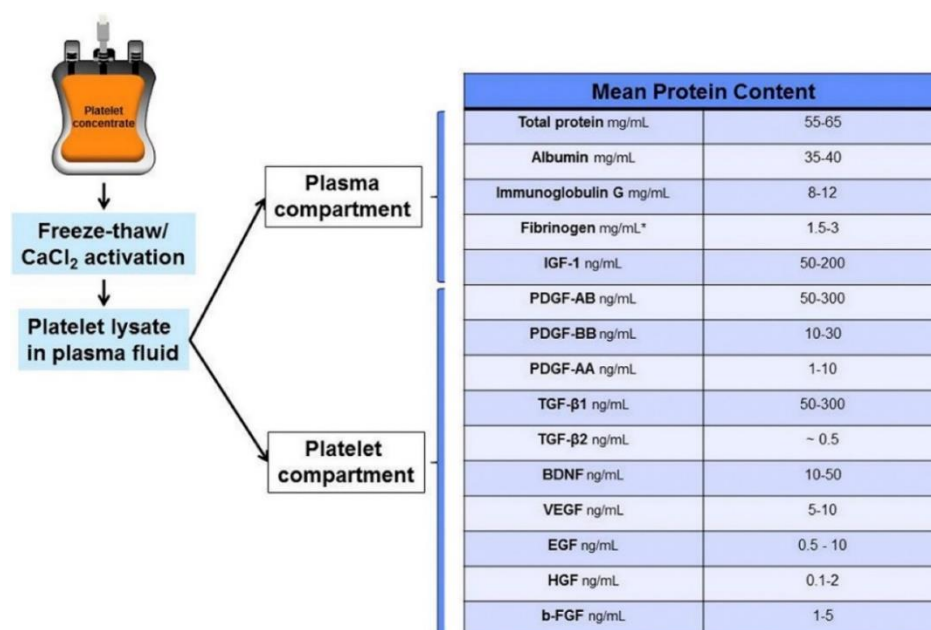


Figure 1.9. Typical protein and growth factor content of human platelet lysate (HPL), adapted from Burnouf et al. (2016).¹⁷⁶

1.4.4 Microstructure of hydrogels

Mechanical characterisation of hydrogels (i.e. rheology) provides useful information without considering the importance of hydrogel ultrastructure. The network possibly identified in the chemical gels were described by Oyen.¹⁸¹ In the chemical gels, the polymer chains can be cross-linked by covalent bonding. Oyen categorised the networks developed from the polymer chains into three types: (1) ideal cross-linked network, (2) non-ideal cross-linked network, with the presence of polymer chain self-loops and free ends and (3) a double network gel showing two distinct networks.¹⁸¹ As the hydrogels are porous systems in which the pore space is occupied by water. The degree of porosity then may correspond to the microstructure which has a substantial effect on the mechanical properties. In order to determine the porosity of the hydrogels, cryofixation is needed. Cryofixation is of interest to prevent water movement and preserve the native structure of the gels. It is important for the hydrogel systems as hydrogels are fairly stable with respect to temperature. Cryofixation is a rapid process (in milliseconds). Artifacts produced by cryofixation are easy to interpret. Compared to cryofixation, chemical fixation is relatively not suitable as addition of a stain or fixative may affect the nanostructure of such systems.

1.5 Thesis aims and scope of this thesis

The introduction of this thesis has established the influence of formulation factors on the release performance of hydrogel-based delivery systems. The first part (Section 1.2 to Section 1.3) outlined the use of hydrophilic matrices for extending the release of drugs. The behaviour of a commonly used polymer, HPMC, in governing the dissolution mechanism of drugs from tablets is emphasised. Several formulation factors impacting the performance of tablets have been reviewed including factors relating to drugs, HPMC and other excipients. A substantial amount of research has recognised the importance of HPMC factors. The present applications of imaging and other microstructural analytical tools in the pharmaceutical field are also described. In Section 1.4, the focus is shifted to more advanced hydrogel systems for a biomedical application, specifically *in situ* forming hydrogels. The dissolution of active substances from the hydrogels, on the other hand, has not been extensively studied.

As highlighted in this introduction, the connection between the formulation parameters and performance of two hydrogel-based delivery systems has been outlined. However, previous studies have not attempted to fully investigate any scientific rationale to evidence any correlation between formulation parameters and release behaviour. The main aim of this study is to demonstrate the formulation-microstructure property relationships of pharmaceutical tablets and hydrogels through the applications of imaging techniques. Initially, a number of analytical techniques will be employed in Chapter 3 to characterise hydrophilic matrix tablets and their raw materials. The microstructure of caffeine tablets will be investigated as a function of polymer content and particle size (Chapter 4). In Chapter 5, other relevant issues regarding the release mechanism will be highlighted, including the 3D percolating networks of HPMC and effect of dissolution on the distribution of HPMC. The final result chapter (Chapter 6) will assess the nanostructure of *in situ* gelating hydrogels based on different gel monomers. The specific objectives of each topic can be found as a preface in Chapters 3 – 6.

Chapter 2:

Materials, experimental techniques and statistical procedures

This chapter provides an overview of materials, analytical approaches and data processing employed in this study. Background of the techniques is briefly discussed in this chapter. Comprehensive details of materials and methods are described in the materials and methods sections of Chapter 3 to Chapter 6.

2.1 Materials

2.1.1 Caffeine

Caffeine (Sigma-Aldrich, UK) was used as a model drug due to a number of reasons. It is widely used in pharmaceutical preparations with paracetamol and aspirin to achieve a synergistic interaction for the treatment of analgesic and antipyretic. It is a BCS class I drug substance with high solubility and high permeability. It is sparingly soluble in water at a temperature between 15° and 25°C (solubility is between 30 to 100 ml of solvent per 1 g of solute).⁹⁶ At 25°C, the solubility of caffeine is pH-independent over the pH range between 2 to 10.5.¹⁸² Caffeine also shows good UV absorption characteristics ($A = 1.224$ at the maximum wavelength $\lambda_{\max} = 272$ nm).¹⁸³ The drug loading content of 10% w/w was used to ensure dissolution sink conditions. The ToF-SIMS characteristic peak of caffeine was found in previous work.¹⁸⁴ The density of caffeine is 1.23 g/cm³ at 18 °C.¹⁸⁵

2.1.2 Hydroxypropyl methylcellulose

Hydroxypropyl methylcellulose (HPMC) 2208 MethocelTM CR Premium (Colorcon, Dartford, UK) was used as a main hydrophilic polymer in this study. The only viscosity of HPMC 2208 selected was Methocel K4M with reported methoxyl and hydroxypropoxy substitutions of 22.7 and 8.6 % respectively.

HPMC K grade was used as it rapidly develops the gel barrier and produces the slowest release among all Methocel grades, contributing to the controlled release of drugs.¹⁶ The apparent density of MethocelTM ranges from 0.25 to 0.70 g/cm³.¹⁸⁶

2.1.3 Lactose

Lactose monohydrate (Lactose Fast Flo 316, Foremost Farms, US) was chosen as a diluent in this study. This product is a spray-dried mixture of crystalline and amorphous lactose. Spray-dried lactose is widely used as a filler and diluent in tablets and capsules¹⁸⁷ as it is inert, cost-effective and has good batch-to-batch consistency.¹⁸⁸ The benefit of the spray-dried lactose over the conventional lactose is that spray-dried lactose can be used as a filler-binder for direct compaction.¹⁸⁸⁻¹⁸⁹ It can also produce the tablet with less friability than the conventional lactose.¹⁸⁹ It is freely soluble in water at a temperature between 15° and 25°C (solubility is between 1 to 10 ml of solvent per 1 g of solute).⁹⁶ Previous research reported that lactose was identifiable using ToF-SIMS as it gave a strong signal at m/z 365 of the molecular ion cationized by Na the positive ion mode ToF-SIMS spectra.¹⁴³

2.1.4 Microcrystalline cellulose

Microcrystalline cellulose (Avicel PH102, FMC Biopolymer, Ireland), as abbreviated as MCC, was used to provide a number of functions in the hydrophilic matrix formulations. It serves as a binder/diluent, provides disintegrant properties¹⁹⁰ and improves drug content uniformity, especially in low-dose API formulations¹⁹¹. MCC is generally regarded as a relatively nontoxic and non-irritant material.¹⁹⁰ It is practically insoluble in water.⁹⁶ Avicel PH102 has never been identified using ToF-SIMS. However, Avicel PH101 rather represented in the low mass hydrocarbon fragments than the unique high-mass fragments in both positive and negative ToF-SIMS spectra.¹⁴³

2.1.5 Magnesium stearate

Magnesium stearate (MgSt) is a mixture of solid organic salts of fatty acids, containing magnesium stearate and magnesium palmitate.¹⁹²⁻¹⁹³ It was used as a lubricant to reduce adhesion between the tablet surface and the die wall during tablet compression. MgSt is added as the last excipient in small amounts of approximately 0.5 – 1 %w/w¹³² in the manufacturing to avoid over lubrication which has a deleterious effect on tablet disintegration and dissolution.

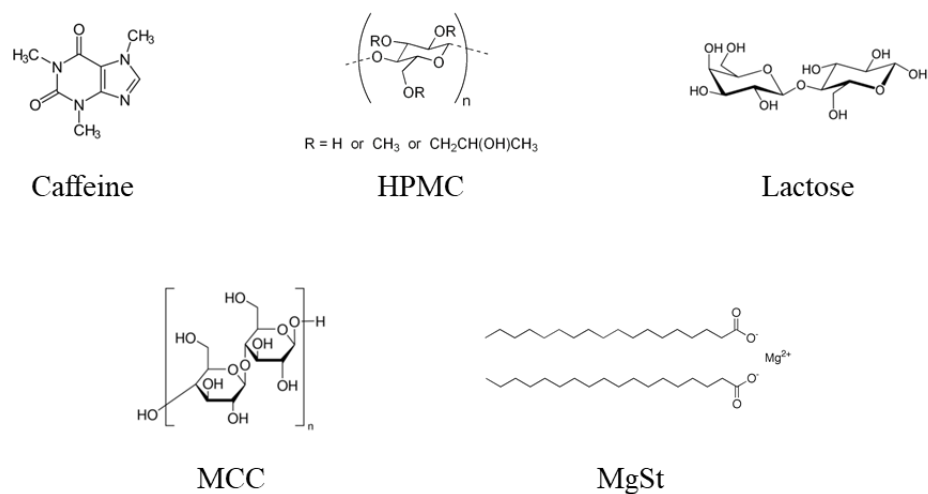


Figure 2.1. Chemical structure of compounds in caffeine tablets.

2.1.6 Polysaccharide-tyramine conjugates

Hyaluronic acid-tyramine (HA-TA) and dextran-tyramine (Dex-TA) conjugates were kindly provided by Hy2Care (Enschede, the Netherlands). HA-TA conjugates were synthesized with a degree of substitution of 16 tyramine groups per 100 carboxylic acid group (%DS = 16). Dex-TA conjugates synthesized by Hy2Care consisted of 10 and 16 tyramine groups per 100 anhydroglucosidic rings (%DS = 10 and 16).

2.2 Experimental techniques

2.2.1 Tablet manufacturing

2.2.1.1 Sieve analysis of HPMC powder

Particle size fractions of HPMC were obtained using a mechanical sieve shaker the (Copley Scientific, UK) with total agitation time of 20 minutes. Fractions of HPMC were achieved using sieves of mesh size 125 μm , 90 μm , 63 μm and 45 μm (Endecott, UK).

2.2.1.2 Formulation and manufacture of HPMC tablets

The amount of caffeine was fixed at 25 mg and the ratio between lactose and MCC was kept constant at 2:1 in all batches. The detailed composition of each batch in a concentration and particle size series is shown in Table 2.1 and Table 2.2 respectively. In the batch sizes of 60 - 100 g, caffeine and all excipients except MgSt were mixed in a 500 mL amber glass jar for 15 min using a TURBULA® mixer T2F (Willy A Bachofen, Switzerland) with a rotation speed of 47 RPM. 0.5%w/w of MgSt was added as the final step into the jar to improve lubrication and then the powders were mixed further with the same rate for 2 min. The powder mixtures were compressed at 150 MPa using a Riva Piccola multi-station press (Riva S.A., Argentina) using 8 mm flat, round tooling (I Holland, Nottingham, UK) with the targeted weight of 250 ± 5 mg. Matrices were manufactured with turret head speed of 10 RPM and feeder speed of 15 RPM. This method was optimised and finalised by Mason (2016) due to its acceptable reported relative standard deviation (RSD) of blend uniformity testing.¹⁹⁴ The thickness of the finished tablets was 4 mm.

Table 2.1. Composition and quantity of each component (%w/w) in caffeine tablets in a concentration series.

Ingredient	Batch					
	1	2	3	4	5	6
Caffeine	10.0	10.0	10.0	10.0	10.0	10.0
HPMC (<125 μm)	7.5	12.5	15.0	17.5	20.0	30.0
Lactose	54.7	51.3	49.7	48.0	46.3	39.7
MCC	27.3	25.7	24.8	24.0	23.2	19.8
MgSt	0.5	0.5	0.5	0.5	0.5	0.5

Table 2.2. Composition and quantity of each component (%w/w) in caffeine tablets in a particle size series.

Ingredient	Batch				
	1	2	3	4	5
Caffeine	10.0	10.0	10.0	10.0	10.0
HPMC	15.0	15.0	15.0	15.0	15.0
Particle size (μm) of HPMC, fractioned by sieve analysis	<45	45-63	63-90	90-125	>125
Lactose	49.7	49.7	49.7	49.7	49.7
MCC	24.8	24.8	24.8	24.8	24.8
MgSt	0.5	0.5	0.5	0.5	0.5

2.2.2 Dissolution testing

2.2.2.1 Determination of caffeine release kinetics from tablets

Dissolution tests were carried out in a volume of 900 mL of de-gassed, de-ionized water using a USP II (paddle) apparatus (Dissolutest, Prolabo, UK). The temperature was maintained at 37 ± 0.5 °C at a paddle speed of 50 RPM. Tablets were held in a sinker to prevent floating or adhesion to the dissolution vessel

wall. Samples were removed from the dissolution vessel at intervals (at 4, 6, 8, 10, 15, 30, 45 and 60 minutes) by a peristaltic pump (60 s pump time) and filtered through 10 μm ultra-high molecular weight polyurethane filters (Copley Scientific, UK). The samples then were flowed into 10 mm flow-through quartz cells for the spectrophotometric determination of absorbance at a wavelength of 273 nm using an 8453 Agilent UV/Vis spectrophotometer system equipped with Chemstation software version 08(03) (Agilent, USA).

2.2.2.2 Comparison dissolution profiles using similarity factor

The similarity factor (f_2), proposed by Moore and Flanner (1996)¹⁹⁵, was adapted to compare dissolution between an experimental dissolution profile and a predicted dissolution profile. The equation of f_2 is shown in Equation 2.1, where n is the number of time points, R_t is the dissolution value of the reference batch at time t , and T_t is the dissolution value of the test batch at time t . To ensure sameness or equivalence, f_2 values should be greater than 50 (50 – 100).

Equation 2.1. The similarity factor.

$$f_2 = 50 \cdot \log \left\{ \left[1 + (1/n) \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \cdot 100 \right\}$$

2.2.2.3 Mathematical models of dissolution profiles

Mathematical models can be used to describe the dependence of release as a function of time, measure physical parameters, enable quantitative interpretation and predict the release of the active agent from the dosage form. There are two dissolution models used to analyse the dissolution data in this thesis, including zero-order¹⁹⁶ and first-order¹⁹⁷ models. Both models were proposed for quantitative evaluation of *in vitro* release behaviour of proteins from the hydrogel.

For a zero-order model, a drug is released from a dosage form at a constant rate and independent of the concentration of the active substance.¹⁹⁸ It results in a straight line plotted between the fraction of the dissolved drug against time.

Zero-order may be aimed for controlled release systems. Zero-order release kinetics occurs when the pharmaceutical dosage forms do not disaggregate and have a very slow drug release (assuming that area does not change and no equilibrium conditions are obtained).¹⁹⁸⁻¹⁹⁹ The zero-order drug release can be represented by Equation 2.2, where F is the fraction (%) of drug released in time t and k_0 is the zero-order release constant.²⁰⁰ Examples of systems that could display the zero-order release rate are transdermal systems, slow release matrix, coated, and osmotic systems.¹⁹⁸

Equation 2.2. Zero-order kinetics.

$$F = k_0 t$$

First-order kinetics can be described in the systems that change in concentration with respect to change in time is dependent only on concentration. Thus, the amount of active released tends to decrease as a function of time. The equation of first-order kinetics can be found in Equation 2.3, where F is the fraction (%) of drug released in time t and k_1 is the first-order release constant.²⁰⁰ An example that follows this type of release model is a porous matrix containing soluble active agents.²⁰¹

Equation 2.3. First-order kinetics.

$$F = 100 \cdot (1 - e^{-k_1 t})$$

An add-in program for Microsoft Excel, DDSolver, was used for modelling drug dissolution profiles with the zero-order and first-order kinetics modelling.²⁰⁰

2.2.3 Optical profilometry

Optical profilometry is an analytical technique enabling topographical data extraction from a sample's surface. Such information includes surface morphologies, step heights, surface roughness, coating thickness and 3D imaging. Generally, profilometry techniques can be categorised into two methods, contact and non-contact. The optical profiler used in this thesis is a structured grid confocal microscope which is the non-contact method based on

light. Technically, the optical profiler is performed by scanning the sample vertically (i.e. moving along the Z-axis). In this study, tablet samples were imaged and measured as a function of depth using optical profilometer (ZDot™, Zeta Instruments, Canada).

2.2.4 Scanning electron microscopy

SEM is a type of electron microscopy widely applied in the field of material sciences for understanding the structures and textures of materials. This technique can image surface topography with a magnification power of 20 - 130,000 $\times$.²⁰² It functions by the irradiation of a finely focused beam of electrons on the surface of the specimen. When accelerated electrons interact with the constituent atoms in the specimen, two main processes occur, the emission of secondary electrons (SEs) and the generation of backscattered electrons (BEs). The emission of the valence electrons of the constituent atoms can generate the SEs. This process is associated with low-energy electrons produced from the superficial regions of the specimen. In contrast, BEs are high-energy electrons which mainly correspond to the inside of the sample.

There are a number of factors needs to adjust to gain a high-quality image.²⁰³ A vacuum is required in SEM settings as the electrons make less contact with any gas molecules so that the vacuum environments would avoid interaction between them which could lead to a poor-quality image. Accelerating voltage is an important part of adjusting image resolution. Lowing accelerating voltage can decrease the production of scattered BEs, hence reduced signal and resolution. Coating the sample with a conductive layer is required in the analysis of nonconductive samples in order to protect the sample from overheating and to improve topographic contrast.

Raw materials and tablet samples were examined using an SEM (JSM-6490LV, JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 5-10 kV in the high vacuum mode with data subsequently analysed using JEOL SEM software.

2.2.5 Cryofixation

Due to the nature of hydrogel systems that contain a large amount of water, liquids in the gels tend to evaporate in the vacuum of an electron microscope system. As chemical fixation can introduce a stain or fixative into the systems which possibly leads to chemical alteration, cryofixation was preferred. The main aim of cryofixation is to preserve the native 3D structure by freezing the specimen almost instantaneously in order to achieve true vitrification (amorphous ice) and avoid visible ice crystals. There were two methods of freezing applied in this research, plunge-freezing and metal mirror (slam freeze) fixation.

2.2.5.1 Plunge freezing method

For cryo-TEM (Section 2.2.5), the plunge freezing method was done by rapidly plunging the hydrogel specimen into liquid ethane to achieve a vitreous film. Prior to that, a cryogen and holey carbon grid needed to be prepared. Ethane, the cryogen, was cooled and maintained in liquid nitrogen (below $-190\text{ }^{\circ}\text{C}$). Liquid nitrogen was chosen to be the first coolant for liquefying ethane as it is readily available and relatively inexpensive. However, its thermal conductivity is only about 400 K/s which may lead to the formation of crystalline ice. The thermal conductivity of liquid ethane is $300 - 400$ times higher than liquid nitrogen. The grid was glow-discharged to turn the surface of the grid to become more hydrophilic so that the gel can consistently spread across the surface and be blotted to form films. The solution of gel samples ($3\text{ }\mu\text{l}$), as detailed in Section 6.3.7 was dropped onto the surface of the carbon grid. Then the liquid droplet is blotted with filter paper for a few seconds. The blotting time was varied from $1.5 - 3.5$ seconds. Consequently, the sample-loaded grid was plunged into liquid nitrogen.

2.2.5.2 Metal mirror fixation

For cryo-FIB-SEM (Section 2.2.7), hydrogel samples were frozen by bringing the hydrogel into rapid contact with a polished cooled metal surface. The well-frozen layer of the hydrogel specimens achieved was physically flat and parallel

to the specimen metal support. Compared to plunge freezing, the depth of ice-crystal free zone with metal mirror fixation is greater and the cooling rate is also higher.

2.2.6 Cryo-transmission electron microscopy

TEM is a type of electron microscopy widely applied in the field of material sciences to study morphological information and internal structure of materials. In TEM a thin specimen (< 100 nm) is required for the electron beam to transmit. The scattered electron is then focused. Generally, there are two modes of TEM operation depending on the size and location of the aperture, diffraction and imaging modes.²⁰⁴ TEM images corresponding to a projection of the materials in the direction of the electron path can be obtained. Due to the high vacuum in the TEM and high degree of water contained in most biological samples, the shrinkage of the samples and supramolecular motion of the structure may be observed without fixation. A frozen-hydrated state of most biological sample needs to be prepared. Vitrification of the sample also reduces the visible effects of radiation damage.²⁰⁵ Details in cryofixation techniques are shown in Section 2.2.5. Anti-contamination devices in the TEM usually used to avoid deposition of materials on the specimen.

Gel samples were analysed under cryogenic conditions using a TEM (JEOL 2100F, JEOL, Japan) at 200 kV equipped with a 914 cryo-tomography holder system (Gatan, Inc, Pleasanton, CA). Sample preparation method can be seen in Section 6.3.7.

2.2.7 Cryo-focused ion beam scanning electron microscopy

Generally, cryo-ultramicrotomy is used to acquire the internal structures of materials. However, an issue concerning visible cutting artefacts made by cryosectioning was described.²⁰⁶ Cryo-focused ion beam scanning electron microscopy (cryo-FIB-SEM) was recently introduced as an alternative to cryo-ultramicrotomy for precise cross-sectioning the specimen.²⁰⁷⁻²⁰⁸ cryo-FIB-SEM was used to provide micron and sub-micron size microstructural illustration of soft matter specimen.²⁰⁸

The ultrastructure of hydrogel samples were investigated using a FEI Quanta 200 3D Dual Beam FIB-SEM (Thermo Fisher Scientific, Massachusetts, United States) coupled with a PP2000 Cryo-SEM Preparation System. After preparing the specimen using a metal mirror fixation (Section 2.2.5.2), the hydrogel was then transferred under vacuum to the ‘cold stage’ of the ‘preparation chamber’ and sputter-coated with platinum. Consequently, the sample was milled with Gallium (Ga) beam. The ion beam generated was used for imaging and/or to mill into the specimen while the SEM analyses each fresh layer and/or the milled face. Following that the SEM holder was transferred to the ‘prep stage’ for the sublimation of water by increasing the temperature to -90 °C for about 30 minutes. Finally, the sample was loaded under vacuum into the SEM chamber once again to analyse.

2.2.8 Confocal Raman microscopy

2.2.8.1 Raman scattering

‘Raman scattering’ or the ‘Raman Effect’ is the inelastic scattering of photons as a result of the interaction between excitation light and molecules. During the scattering process, the scattered photons may lose energy, thereby having longer wavelengths than the incident photons (Stokes Raman scattering). On the other hands, the kinetic energy of protons may be increased resulting in decrease in wavelengths (anti-Stokes Raman scattering). Inelastic scattering occurs less prominently compared to elastic scattering (Rayleigh scattering) which scattered photons have the same frequency as the incident photons. Raman spectroscopy measures molecular vibrations similar to FTIR spectroscopy. The findings from both techniques are complementary to each other. However, Raman-active molecules require a change in polarizability, whereas, FTIR spectroscopy depends upon a change in the dipole moment due to the interactions between a sample and absorbed light. Water has a strong dipole moment demonstrating an intense IR spectrum. Therefore, Raman spectroscopy is less interfered by water compared to FTIR, while water cannot be used as a solvent in FTIR measurements. Nonetheless, fluorescence (caused by intrinsic or impurities)¹²⁴ may strongly overlay the Raman bands.

2.2.8.2 Basic principles of confocal Raman microscopy

A Raman spectrometer was first merged with an optical microscope in the 1990s.²⁰⁹ In general, the sample is illuminated with a point source which is passed through a pinhole in front of the detector. The image of the sample can be obtained by three methods. ‘Point-by-point Raman mapping’ is created by raster scanning the sample at a given number of sample position. Instead of illuminating a single point, ‘Line imaging’ illuminates an entire line across the sample area (one dimension) enabling collecting hundreds of spectra at the same time. The last method ‘global imaging’ illuminates the sample as a whole (two dimensions) by probing the light through a narrowband filter onto a detector. As the pinhole limits the signal detected, the light above or below the focal plane cannot reach the detector. Therefore, the image is only contributed by the light from the focal plane. Raman scattering is intrinsically weak making it even harder to detect in confocal Raman microscopy (CRM). Raman-active molecules need to be able to change in polarizability; this means the ability of the electron cloud of molecules can be distorted when they are in an electromagnetic field.²¹⁰ More basic fundamentals of Raman spectroscopy are mentioned in Section 1.3.3.4.

2.2.8.3 Data analysis

Data analysis includes data pre-processing and processing. WITec Project FOUR software (WITec Inc., Ulm, Germany) was utilised for analysing the Raman hyperspectral data. The objective of data pre-processing was to minimise the background noise before the generation of images. Data pre-processing in this research comprises cosmic ray removal and background subtraction. Charge-coupled device (CCD) detectors, which are used widely in a typical Raman spectrometer, are susceptible to cosmic rays demonstrating randomly visible sharp peaks in Raman spectra. Cosmic ray removal was used to detect and filter the cosmic spikes from the spectra. Fluorescence usually interferes Raman scattering resulting in a non-linear background. Noises could also be originated from the CCD camera. In order to reduce the effect of fluorescence and other sources of noises, background subtraction was applied.

Image generation can be done by two methods, univariate and multivariate data analysis. Univariate analysis evaluates one spectrum at a time, whereas, multivariate analysis considers all spectra in the data set.²⁰⁹ In this study, two multivariate analysis algorithms were employed, Basis analysis and K-means cluster analysis. Due to a *priori* knowledge, Basis analysis, provided by WITec Project FOUR software (WITec Inc., Ulm, Germany), was performed in order to relate the detected spectra from caffeine matrices to spectral information of raw materials. However, it is crucial to ensure that the pure spectra are present in the sample. Care must be taken when the chemical reactions or mixed spectra of components might have been found. Another point to note is that the reference spectra are not always available. Unsupervised K-means cluster analysis partitions spectra into clusters. Prior to the analysis, the number of clusters (K) is selected. The algorithm initially works by calculating K centroids. Each observation (the Raman spectrum) is then assigned to the group of centroids according to their closest distance. The position of centroids is re-calculated. Again, the spectra are categorised to their new group based on the distance to the calculated centroids. The procedure is repeated until all assignments cease to change.

2.2.9 Time-of-flight secondary ion mass spectrometry

2.2.9.1 Basic principles of time-of-flight secondary ion mass spectrometry

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface-specific mass spectrometry technique in the domain of SIMS. The initial idea of ToF-SIMS emerged in a group led by Benninghoven in late 1960.²¹¹ There is several possible processes continually occurs in SIMS instruments. First of all, the primary ion source, such as a liquid metal ion gun/source (LMIG/LMIS), generates a pulsed, mass-separated primary ion beam with high energy (between 1 and 25 keV²¹²). As illustrated in Figure 2.2A, the primary ion beam is focused onto a solid surface causing primary ion bombardment. Consequently, the kinetic energy of the primary ions is transferred to the atoms of the solid at or close to a surface via the billiard-ball-type collisional process.²¹² The high energy of the primary ion beam also leads to a ‘cascade’ of collisions between

the atoms themselves. If the atoms or group of atoms obtain sufficient energy (typically 20 to 40 eV)²¹² which is higher than the binding forces of the species in the surface, the emission/sputtering of ‘secondary’ particles will occur. The sputtered secondary particles can be predominant neutrals and positive or negative secondary ions. These secondary ions liberate from the surface with a range of kinetic energies. For mass analysis, the secondary ions need to be accelerated to the same nominal kinetic energy so that they enter the field-free drift section of a flight tube. The flight time of the secondary ions is, therefore, dependent upon their mass. The time increases with the mass of the ions.

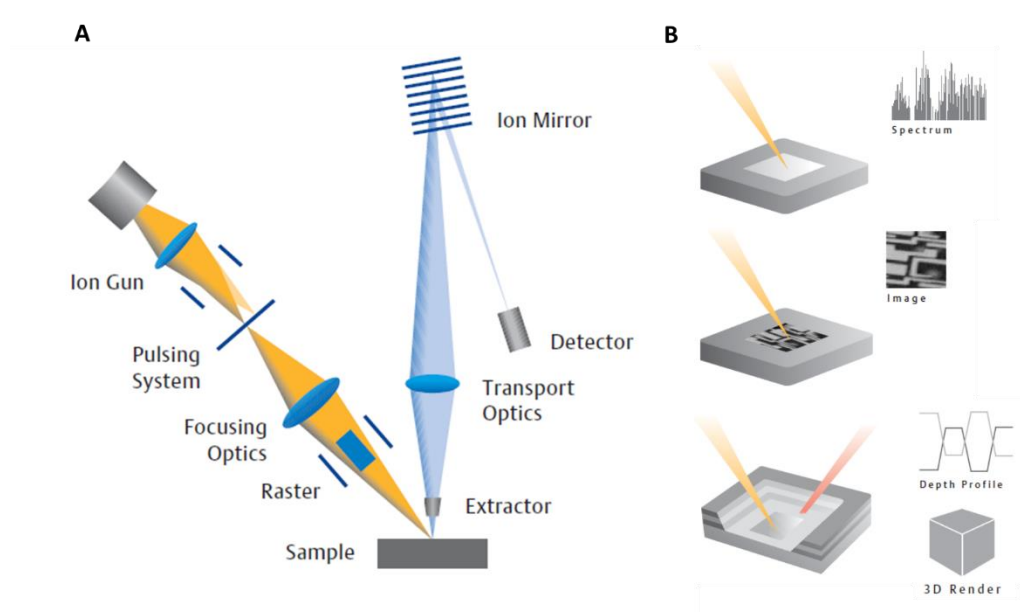


Figure 2.2. Schematic diagram showing (A) basic principle of ToF-SIMS and (B) modes of operation: surface spectroscopy, surface imaging and depth profiling.²¹³

2.2.9.2 Modes of operation

Generally, SIMS can be categorised into two modes, static and dynamic SIMS. It primarily depends on the dose of the primary ion beam used during analysis. In terms of mode of operations, ToF-SIMS can be applied for four different functions, including surface spectroscopy/static SIMS, surface chemical imaging, depth profiling and 3D chemical imaging. The difference between static and dynamic SIMS, along with a brief overview of four different modes

of operation, are given. Figure 2.2B shows the opportunity of SIMS employed in different modes of operation.

Owing to primary ion bombardment, the sample surface may be modified by emitted secondary ions. Static SIMS utilises an extremely low primary ion dose which is maintained within the 'static limit'. This limit is described as the primary ion dose at or below 10^{12} to 10^{13} ions/cm². Under this condition, less than 1 % of the top sample layer of atoms or molecules is affected by the primary ions. With regard to such a low ion dose, the interaction of the primary ions with the sample surface is considerably low. The removal of liberated species shows a non-destructive effect on the vast majority of the sample surface keeping a sample in a non-destructive mode.

Under the static conditions, the ToF-SIMS instrument can be used to provide SIMS spectra which correspond to the separation of emitted secondary ions according to their mass-to-charge ratio (m/z). Surface spectroscopy can demonstrate the capability of tracing elements and obtaining isotopic compositions.

In addition to acquiring mass spectra of a single coordinate of the primary ion beam spot, the mass spectra of a particular region can be scanned and analysed. During ToF-SIMS analysis, the spatial coordinates of each pixel are recorded by moving the primary ion beam to a neighbouring pixel. The new pixel is then focused by the short pulse of the primary ion beam. A spatially resolve single ion image/map can be reconstructed. The total ion image comprising all secondary ions of significant intensity can also be generated. There are a number of advantages of imaging SIMS over other existing imaging techniques, e.g. fluorescence microscopy and atomic force microscopy. ToF-SIMS imaging offers high resolving power, sensitivity, chemical selectivity with required minimal or no sample preparations.²¹⁴ ToF-SIMS chemical mapping is useful to evaluate the uniformity of multicomponent systems.

For several systems chemical information in the underlying layers beneath the surface is also crucial. ToF-SIMS is capable of gaining compositional

information varied with depth and rendering chemical distribution in 3D. Compared to XPS depth profiling, ToF-SIMS offers greater spatial and depth resolutions²¹⁵, molecular specificity²¹⁵ and high mass resolution²¹⁵. For such functions, ToF-SIMS implements a dual-beam which combines a ‘sputter ion beam/gun’ to an ‘analysis beam/gun’. The sputter gun (e.g. SF_5^+ and argon gas cluster ion beam; Ar-GCIB) is utilised for gentle eroding the sample surface to form a sputter crater. Initially, the predefined area is rastered over by the sputter ion beam. The dose of the sputter ion beam is substantially higher than the analysis beam and exceeds the so-called static limit. As a consequence, the sample surface was etched away. It is worth noting that removing a large volume of the sample may alter or damage the analysed area leading to the change in ion yields. Especially organic and biological samples which are relatively more sensitive than inorganic materials, it is paramount that the samples are maintained as closely to their native state as possible. There have been recent attempts to develop appropriate sputter ion beams for depth profiling and 3D imaging. The details for this are discussed in Section 2.2.9.3. After the sputtering event, the analysis primary ion beam irradiates an analysed area which is located inside the sputter crater and typically smaller than the size of the sputtering area. The resulting secondary ions are extracted and collected into the mass analyser, continually the sputtering area is exposed to the subsequent pulse of sputter beam enabling the next round of analysis by the primary ion beam. This step is repeated until the required depth is reached. A depth profile can be produced by selecting secondary ions of interest from the mass spectra and comparing the intensity of those ions as a function of depth or increasing sputtering time. Depth profiling allows the evaluation of the varied composition of chemicals as a function of depth. Another mode of ToF-SIMS operations which can be done using dynamic SIMS is the 3D visualisation of chemicals. Secondary ion images showing the lateral distribution of chemicals of interest can be combined with depth profiling data to generate the 3D mapping. This function allows the reconstruction of 2D secondary ion images with different planes of the recorded 3D data cube including the XY, XZ and YZ planes.

2.2.9.3 Primary ion beam systems

The ToF-SIMS instrument consists of four main components: a primary ion source, an ion-optic lens system, a time-of-flight (ToF) mass spectrometer and a detector. The primary particle source generates the pulses of primary ion beams with a range of beam energy (10 to 1000 eV)²¹². Over a few decades, primary ion beam sources have progressed from originally monoatomic sources such as Ar^+ , Xe^+ , Ga^+ to more recently and widely used polyatomic sources such as Au_3^+ , Bi_n^{p+} ($n \geq 2$ and $p \geq 1$). These sources have been developed along with alternatively larger cluster sources such as Buckminsterfullerene (C_{60}^+) and Ar_n^+ ($500 \leq n \leq 2500$)¹³⁴. The most notable advancement is that the cluster ion beams cause less detrimental effects on soft materials such as organic, polymeric and biological samples than monoatomic sources.

Even though the monoatomic primary ions could provide chemical specificity, the issue regarding monoatomic primary ion beam was that these ions destructed the sample's surface. Thus the analysis had to be performed under the so-called static limit at which the primary ion dose is maintained (10^{12} to 10^{13} ions/cm²). Under such conditions, the impact of primary ions on the surface is low and causing less than 1 % of the surface to remove. Accordingly, the detected secondary ions should be originally emitted from the uttermost surface. With regard to low primary ion dose, the number of secondary ions emitted and detected per primary ion impact or secondary ion yield is limited. This low dose of ions makes it impractical to depth profile the sample.²¹⁶ Apart from that, the efficiency of sputtering the secondary ions with m/z 250 or more is low so that there is a problem with the size of analysed molecules.²¹⁶

Polyatomic molecules and metal clusters have received increased attention as primary ion beam systems since 1989. Initially, the LMIG technology was based on gallium before it was changed to gold and then bismuth. LMIG sources can generate highly focused ion beams (FIBs) from a very fine tip of a needle in a Taylor cone contributing to submicron lateral resolution for chemical imaging. The efficiency of ToF-SIMS measurements (e.g. secondary ion yields) could be improved extensively using gold primary clusters²¹⁷ (e.g. Au_3^+). Nevertheless,

the weakness of the gold LMIG is that Au_1^+ predominates when gold ions are created rather than preferable cluster ions such as Au_2^+ or Au_3^+ . In 2004, bismuth was used to replace gallium and gold and it was becoming the standard primary ion source. The previous study led by Touboul demonstrated that ToF-SIMS images obtained with the Bi_3^+ ion beam were better in lateral resolution with a wider accessible mass range than with the Au_3^+ gold LMIG²¹⁸ so that the Bi_3^+ ion beam is recommended for large-area imaging. It was due to the increase of primary ion intensity with Bi_3^+ which also led to a reduction in data acquisition time by comparison with Au_n^+ .²¹⁸ It was found that there was a strong yield enhancement with an increasing number of constituents for molecular primary ions such as SF_5^+ , $\text{C}_{10}\text{H}_8^+$, and C_6F_6^+ .²¹⁹ By comparison with focused Ar^+ primary ion beam, SF_5^+ demonstrated superior imaging capabilities when mapping characteristic secondary ion emission from a model sample system containing polypropylene.²¹⁹

In conjunction with LMIG sources, large cluster ions have also been employed in SIMS particularly for the depth profile analysis of organic materials. The main advantage of such primary ions is that the sample is less destructive. The examples of available cluster sources used in SIMS include SF_5^+ , C_{60}^+ and other C-based sources, Au_n^+ , Bi_n^+ and gas cluster beams²²⁰. There is a considerable amount of research on depth profiling with gas cluster ion beams (GCIBs).^{141, 221-226} The GCIBs consist of abundant atoms or molecules of gaseous materials (e.g. Ar, O_2 , CO_2 , or SF_6 gases).²²⁰ It was found in the literature that with the large argon clusters, damage accumulation was reduced as the argon cluster size is increased and the degree of damage accumulated decreased, compared with C_{60}^+ .²²⁷ The sputter rate was approximately constant as a function of Ar cluster size.²²⁷

2.2.9.4 Time-of-flight mass analysers, ion-optic systems and detectors

Typically, a quadrupole or magnetic sector mass analyser is used in conventional SIMS instruments for sampling secondary ions which are sputtered from the surface. These mass analysers, though, are only able to collect a specific small mass interval at any particular time. At best, a few mass intervals can be done in

the case of a magnetic sector mass analyser with a multicollector. A sequential secondary ion beams from different species are then detected by the detector array. Under these conditions, the evaluation of various ions which are out of the selected mass range is inherently impossible making it is not practical for determining sample composition.

ToF mass analysers are built for overcoming this limitation. At any given time ions are produced with a narrow energy range (typically < 20 eV). After flying through a flight tube, the ions arrive at the detector with different time according to their mass, as shown in Equation 2.4, where m is the mass, q the charge, U the extraction voltage, t the time and s the effective flight path length.

Equation 2.4. Mass-to-charge ratio.

$$\frac{m}{q} = \frac{2U}{s^2} t^2$$

There are several ion-optic elements added to the ToF-SIMS instrument for specific purposes. A beam extractor focuses the ion beam to be directed toward the detector. A reflectron, located in the ToF mass analyser, has a region of static fields to reverses the ion path.

Microchannel plate detectors are normally employed in ToF-SIMS instruments. Due to its flat surface, the entering ion flux can be presented with an orthogonal direction. Due to the fact that the light ions arrive earlier than the heavier ions, this dense array of separate electron multipliers can detect multiple ions in the same mass spectrum without degraded capacity. Digitised outputs with pulse counting are employed to produce a mass spectrum from a cycle of primary ion pulses.

2.2.9.5 Common problems associated with ToF-SIMS

Even though ToF-SIMS can provide an accurate qualitative characteristics of samples' surface, it is not quantitative. The ionization probability of the selected molecule is varied with surrounding environments. This phenomenon is called the 'matrix effect'. A research study found that the matrix effects were more

pronounced when targeting higher mass species than lower mass species. The effect of primary ion clusters was found; using larger primary ion source such as Ar_{3000}^+ resulted in more adversely matrix effect than utilising smaller ones such as Bi_3^+ .⁷⁴

The ‘edge effect’ is the variation in sputtering yield and signal intensity due to varying in primary beam angle incidence caused by the topography of the sample.²²⁸ Most commonly, this effect can be resulted by the sputtered crater in dynamic SIMS. It is exemplified by previous studies.²²⁸⁻²³⁰

‘Image shifting’ is one of the most common problems during the acquisition of dynamic SIMS. As the analysis and sputter beams are technically fixed and located at fixed angles to the target sample surface. During depth profiling, the position of the sample is moved laterally concurrently with the removal of a layer of material. The degree of lateral shift mainly depends upon two factors, the angle between the primary ion beams and the sample surface and the depth of the analysis required. In the case of 45° angle of incidence, the image will be shifted laterally in an equal distance as the depth of analysis. A number of methods were recommended for accounting for image shifts such as using software to correct them, adjusting the height of samples by the same amount as the thickness of removed material and adjusting the beam position during data acquisition.²³¹

2.2.9.6 General sample considerations

There are standard requirements for sample preparation that need to be considered. The ToF-SIMS experiment needs to perform in an ultrahigh vacuum chamber (up to 10^{-10} mbar¹³⁴). Therefore, the samples are required to be stable under vacuum conditions. The sample surface exposed to the primary/analysis beam should be flat. Surface roughness involves the process of secondary ion extraction into the mass analyser. If the surface is not flat or not aligned, the sputter rate of the probed secondary ions possibly varies. It results in poor mass resolution. ToF-SIMS is a highly surface-sensitive technique so that any contaminants on the surface could be recorded. The source of contamination

could be possibly from sample preparation procedures, storage containers, transportation of the samples. During sample preparation, the samples may contaminate from dirty tools or even nitrile gloves. Therefore, it is necessary to avoid the contamination of samples such as wearing high-density polyethylene (HDPE) gloves and using clean tools. It is preferable to use a laminar flow cabinet to prevent dust contamination from the air.

As mentioned above, specific types of samples need special handling procedures for SIMS analysis. For the powder samples, the simplest method is to apply the powder samples to a flat substrate material, silicon wafer, for instance. It can be done by using a small piece of clean double-sided tape and gently tapping the powders into the sticky area of the tape. It is recommended to invert the samples to remove excessive powders.²¹² A jet of argon gas or compressed air is suggested to blow away the excess powder. This step prevents the cross-contamination of excessive powder to/from other samples in the sample chamber. For the tablet samples, cross-sectioning may be needed to produce the flat surface. The approaches to preparing cross-sections can be done differently such as microtoming and fracturing.²¹² A diamond-tipped cutting blade may be required for microtoming as the cutting result tends to free from striations.²¹²

2.3 Statistical procedures

Methods of statistics used in this thesis are described with the focus upon correlation and regression analysis.

2.3.1 Hypothesis testing, *t*-test and ANOVA

Statistical tests, including *t*-test and ANOVA, were performed using Graphpad Prism 8.1.2 (San Diego, California, USA).

2.3.2 Correlation analysis

Correlation is a statistical method used to measure a relationship between two continuous variables. A numerical measurement of the strength of that relationship can be expressed by Pearson's correlation coefficient (*r*). The *r*, which ranges from -1 to +1, can be interpreted as shown in Table 2.3. The *r* value of zero indicate that absolutely no linear relationship exists between the two variables, whereas a coefficient of -1 or +1 indicates a perfect correlation. A positive linear relationship ($0 < r \leq 1$) between the two variables exists when the changes of two variables are in the same direction. Contrarily, there is a negative linear relationship or an inverse correlation ($-1 \leq r < 0$) when a change in one variable causes a change in the opposite direction of another variable. Correlation analysis in this thesis was performed using Graphpad Prism 8.1.2 (San Diego, California, USA). The formula for calculating the *r* is shown in Equation 2.5, where *n* is sample size, *x_i* and *y_i* are the individual values indexed with *i* and $\bar{x}$ and $\bar{y}$ are the sample means.

Equation 2.5. Correlation.

$$r = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 \sum_{i=1}^n (y_i - \bar{y})^2}}$$

Table 2.3. Interpretation the magnitude of Pearson's correlation coefficients (r), adapted from Hinkle et al. (2003)²³², Mukaka (2012)²³³ and Scheff (2016)²³⁴.

Value of r	Interpretation
$r = 1.0$	Perfect correlation
$0.9 < r < 1.0$ ($-1.0 < r \leq 0.9$)	Very high correlation
$0.7 < r \leq 0.9$ ($-0.9 \leq r < -0.7$)	High correlation
$0.5 < r \leq 0.7$ ($-0.7 \leq r < -0.5$)	Moderate correlation
$0.3 < r \leq 0.5$ ($-0.5 \leq r < -0.3$)	Low correlation
$0.0 < r \leq 0.3$ ($-0.3 \leq r < 0.0$)	Negligible correlation
$r = 0.0$	Uncorrelation

A correlation coefficient of 0.90 to 1.00 or -1.00 to -0.90 was used as a criterion for selecting a variable to develop simple or multiple linear regression models.

2.3.3 Simple linear regression and multiple linear regression

Regression analysis is a statistical technique applied with attempts to describe the dependence of one or more factors (independent variables) on the variable of interest (the response or dependent variable). The difference between the regression and correlation is that the regression aims to prove a cause-effect relationship between the variables but the correlation only focuses on the strength of the relationship without making the assumption about the dependence of the independent variables on the dependent variable.²³⁴ Simple linear regression can be used as a model to describe the relationship between the single independent variable and the single dependent variable with the equation shown in Equation 2.6, where y is the predicted value of the dependent variable, b_0 is y -intercept, b_1 is the slope of the regression line, x is the value of the independent variable and e is an inevitable error that could occur during the prediction.²³⁵ In the case of multiple regression, more than one independent variables are handled.²³⁶ The equation for the multiple linear regression model can be seen in Equation 2.7. The data consists of k sets of observations $(x_1, x_2, \dots, x_k)$, where the b coefficients are unknown parameters and ε are random error terms.

Equation 2.6. Simple linear regression.

$$y = b_0 + b_1x + e$$

Equation 2.7. Multiple linear regression.

$$y = b_0 + b_1x_1 + b_2x_2 + \cdots + b_kx_k + \varepsilon$$

All the linear regressions were performed using the Excel add-in ‘Analysis Toolpak’.

2.3.4 Model building

The aim of building the model is to forecast the future behaviour of the dependent variable depending on the database of independent variables. There are generally three methods for variable selection in regression models.²³⁷ Forward selection starts with no variables in the model and adds variables to the model one at a time. Once the variable enters the equation it cannot be eliminated.²³⁸ The process will be finished if the last variable entering the model has insignificant regression coefficient or all the variables are included in the model. By contrast with forward selection, backward elimination begins with the model with all of the potential independent variables and successively removes one at a time from the model. The process will be done when all the variables are significant or all but one variable has been deleted. Once the variables have been dropped, they cannot re-enter the equation.²³⁸ Stepwise regression combines the procedures from both forward selection and backward elimination. It considers the level of significance based on the inclusion of variables in the model and the exclusion of variables from the model. It then always re-evaluates each variable in the model. Therefore, stepwise regression was used in this study as an approach to the selection of significant HPMC parameters. The criteria for the linear regression models are the following:

- The model passes F-test with p value of < 0.05 ensuring that the overall model is significant at 95 % confidence interval.

- Each independent variable passes its individual Student t -test with p value of < 0.05 demonstrating that all independent variables in the model are significant at a 95 % confidence interval.
- In the case of simple regression, the coefficient of determination (R^2) is over 0.90 and in case of multiple regression, the adjusted coefficient of determination ($\bar{R}^2$) is over 0.90. This shows that 90 % of the variation in the release data is explained by variations in an independent variable or a set of independent variables.
- The model has the smallest mean absolute error (MAE) demonstrating the smallest errors and thus offering the highest precision.

MAE is a statistical indicator used to assess the mathematical models. Results from the model are estimates or predictions (P_i ; $i = 1, 2, \dots, n$). When they are deducted by pairwise matched observations (O_i ; $i = 1, 2, \dots, n$). The error from the difference of each pair can be defined as $e_i = P_i - O_i$. Calculation of MAE is shown in Equation 2.8.

Equation 2.8. Mean absolute error.

$$MAE = \frac{\sum_{i=1}^n |e_i|}{n}$$

Chapter 3:

Chemical characterisation of tablet formulations: from raw materials to tablets

3.1 Introduction

Even if excipients usually have no pharmacological effect, they have physicochemical properties which may impact efficacy and safety of finished products. To assess the performance of tablet formulations such as assay of drugs, dissolution and content uniformity, conventional analytical methods, including HPLC and UV-vis spectroscopy, are routinely used. However, conventional approaches lack spatial resolution. Acquiring the distribution of tablet components may reveal useful chemical information such as compositional homogeneity and the impact of chemicals on tablet performance. It is necessary to combine a knowledge of the distribution and the chemical composition using a range of techniques extending from simple optical microscopy to those capable of identifying molecules present in systems and even providing atom resolution.

An optical profilometer is a tool used to provide topographical data and measure a surface's profile. This measuring instrument is applicable to samples with low reflectivity or highly mixed reflectivities. In spite of that, optical imaging may be not suitable to distinguish a sample containing similar component morphologies. Applying other analytical techniques with the ability to chemically resolve the components in the system is a further essential step towards thorough microstructural investigations of the pharmaceutical systems. Such techniques including CRM and ToF-SIMS may prove their effectiveness in enhancing the understanding of excipient roles in formulations and how the microstructure of tablets affect the final tablet performance. Distributional homogeneity obtained from chemical imaging can be evaluated by visual inspection of chemical distribution maps. The cross-sectional analysis method is

developed and detailed in this chapter in order to fully provide insights into the interior microstructure of the tablets.

3.2 Aim and objectives

The aim of this chapter is to employ a number of analytical techniques to characterise the caffeine tablets and raw materials. The objectives are:

- (1) Examine the morphology and determine the chemical characterisation of raw materials.
- (2) Assess the internal microstructure and distributional homogeneity of key ingredients in tablets using optical and chemical imaging approaches.
- (3) Determine the correlation between the role of excipients in the formulation and their chemical distribution.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Raw materials

Raw materials of the caffeine tablets comprise caffeine (Sigma-Aldrich, UK), HPMC 2208 Methocel K4M CR Premium USP (Colorcon, UK), lactose 316 NF Fast Flo, MSD, UK, Microcrystalline cellulose Avicel PH 102 (MSD, UK) as abbreviated as MCC and magnesium stearate as abbreviated as MgSt (Sigma-Aldrich, UK).

3.3.1.2 Caffeine tablets

The 25 mg caffeine tablet formulations were optimised and developed by Mason (2017).^{61, 194} The manufacture of this tablet can be found in Section 2.2.1. The tablets contain 15% HPMC with less than 125 µm of HPMC particle size fractions. Other ingredients included lactose, MCC and MgSt. The ratio between lactose and MCC was kept at 2:1.

3.3.2 Sample preparation

3.3.2.1 Preparation of powder samples

Prior to analysing raw materials using SEM, CRM and ToF-SIMS, powder samples were adhered to a 10 mm diameter aluminium stub using double-sided carbon discs for CRM analysis and then sputter coated with platinum for morphological analysis. In the case of ToF-SIMS analysis, the powders were attached to a cleaned silicon wafer by a double-sided adhesive tape. The powder samples were then exposed to a direct jet of argon gas to eliminate excess powder.

3.3.2.2 Preparation of tablet samples

For the cross-sectional analysis of the caffeine tablets using SEM, the tablets were frozen in liquid nitrogen in order to avoid cutting defects prior to cutting

with a razor blade. The cross-sectioned surface was not entirely flat to maintain the morphology of the composition in the tablet. Following that, it was adhered to an SEM stub with a carbon disc in order to be sputter-coated with platinum.

For the cross-sectional analysis of the tablet sample using CRM and ToF-SIMS, One-third of the tablet was removed using a razor blade. The cut face of the tablet was mounted on the top of an aluminium stub using a small amount of resin. The curing method applied depends upon which resin is used, in this work, Vitralit (Panacol, Germany) and Araldite Rapid (Huntsman Advanced Materials, Switzerland) were selected. In the case of Vitralit, the tablet was subsequently exposed to blue light for 5 minutes to induce polymerisation. If Araldite is used, polymerisation may take at least 12 hours to be fully complete at room temperature without the use of the blue light. Following this the tablet was cross-sectioned with a PowerTome XL ultramicrotome (Boeckeler Instruments, US) using a diamond knife (RMC diamond knives, the United States) until it reached the approximate centre of the tablet. The dust produced by microtoming was 'blown out' in order to avoid cutting interruption. The cross-sectioned tablet surfaces were then amenable to analysis by Raman microscopy and ToF-SIMS.

3.3.3 Scanning electron microscopy

The raw materials of the caffeine tablets and the cross sectioned surface of the tablet were examined using an SEM (JSM-6490LV, JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 5-10 kV in the high vacuum mode with data subsequently analysed using JEOL SEM software.

3.3.4 Optical profilometry

To extract the topographical data, the optical images of the cross-sectioned caffeine tablet were obtained using an optical profilometer (ZDot™, Zeta Instruments, Canada) with ×5 and ×20 objective lens.

3.3.5 Confocal Raman microscopy

3.3.5.1 Raman imaging

The Raman measurements in this work were acquired under ambient conditions with an alpha 300 WITec confocal Raman system (WITec Inc., Ulm, Germany), using the 785 nm near-Infrared laser source and a $\times 20$ objective lens. The NIR laser was employed in Raman experiments owing to the reduced fluorescence interference in the MCC spectra compared to the 532-nm laser (Section 8.1). A cross-sectioned tablet surface area of $700 \times 2800 \mu\text{m}^2$ was scanned with an integration time of 0.3327 s and a step size of $10 \mu\text{m}$.

3.3.5.2 Raman data processing and image generation

Raman data was analysed using WITec Project FOUR software (WITec Inc., Ulm, Germany). The steps into spectral pre-processing for minimising the background noise included cosmic ray removal and background subtraction (Section 2.2.8.3). K-means cluster analysis was used to identify similar spectra and classify the multi-spectrum data into a number of clusters (Section 2.2.8.3). The graph de-mixing was performed to subtract or add the spectral information from/to other spectra in order to remove the contribution between the component's spectra. The data was further processed and optimised for visualisation by applying the basis analysis and K-means cluster analysis algorithms²³⁹⁻²⁴⁰. Finally, an image overlay was used to facilitate the correlation of each component image.

Data processing can be done using univariate or multivariate methods. Univariate methods consist in selecting a band of the chemical of interest in the spectral region which is not masked by other component spectral features to determine the spatial distribution of that chemical entity.

3.3.6 Static time-of-flight secondary ion mass spectrometry

3.3.6.1 ToF-SIMS data acquisition

ToF-SIMS spectral and imaging data in this study were performed using a ToF-SIMS IV instrument (IONTOF GmbH, Münster, Germany). The samples were analysed using a 25 keV Bi_3^+ LMIG primary ion source. A flood gun was used to provide low energy electrons (20 eV) for compensating any surface charge. The total primary ion beam dose for each analysed area was kept below 1×10^{12} ions/cm², thereby ensuring static conditions.

3.3.6.2 ToF-SIMS data processing and analysis

Data was primarily acquired in both positive and negative secondary ion polarity modes. Known chemical structures were searched and identified. Additionally, reference samples of HPMC, lactose, MCC and MgSt were acquired to identify and/or confirm characteristic fragments. The tablet sample was raster scanned randomly with an analysed area of $2.2 \times 4.1 \mu\text{m}^2$.

3.4 Results and discussion

3.4.1 Particle morphological characterisation of raw materials using SEM

In order to gain information about the morphology of the powder samples, the characterisation of each component using SEM was performed. The SEM micrographs obtained for caffeine are shown at $\times 230$ and $\times 950$ in Figure 3.1A and B respectively. It can be observed that individual caffeine particles tended to be multiple formed clusters. The SEM in Figure 3.1B (magnified area from Figure 3.1A highlighted by a red box) shows that there was an aggregate of needle-shaped particles in the clusters.

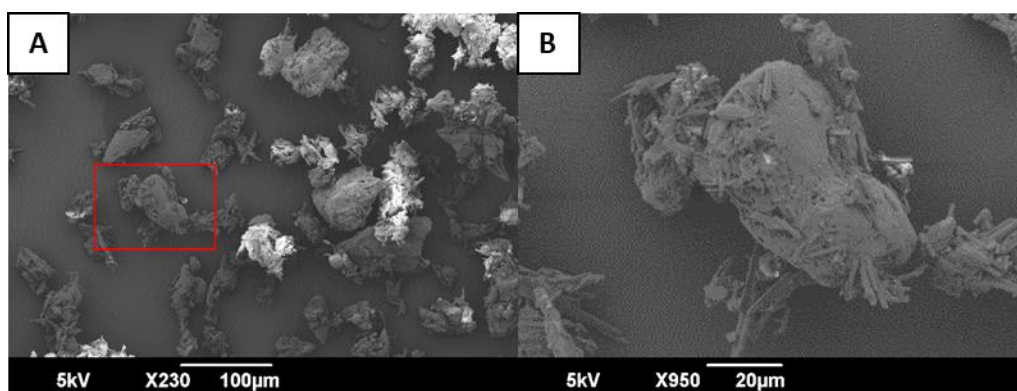


Figure 3.1. SEM micrographs of pure caffeine: (A) at $\times 230$ magnification and (B) magnified part of the area in the red box in (A) at $\times 950$ magnification.

The SEM images of excipients, specifically HPMC, lactose, MCC and MgSt, are shown in Figure 3.2A-D respectively. The morphology of HPMC powder (Figure 3.2A) was long and fibrous which is similar to MCC (Figure 3.2C). This finding highlights that their sizes were in the same range, demonstrating how difficult it would be to differentiate these materials using visual observation. Nonetheless, more agglomerates of the MCC than HPMC particles can be observed. Lactose particles, as shown in Figure 3.2B, particularly exhibited distinct crystalline spherical particles with a rough surface. MgSt particles exhibited very fine, light powders (Figure 3.2D). The SEM findings of these excipients are in agreement with those reported in the Handbook of

pharmaceutical excipients.^{13, 187, 190, 192} The morphological data of raw materials may aid in the identification of components in a matrix tablet.

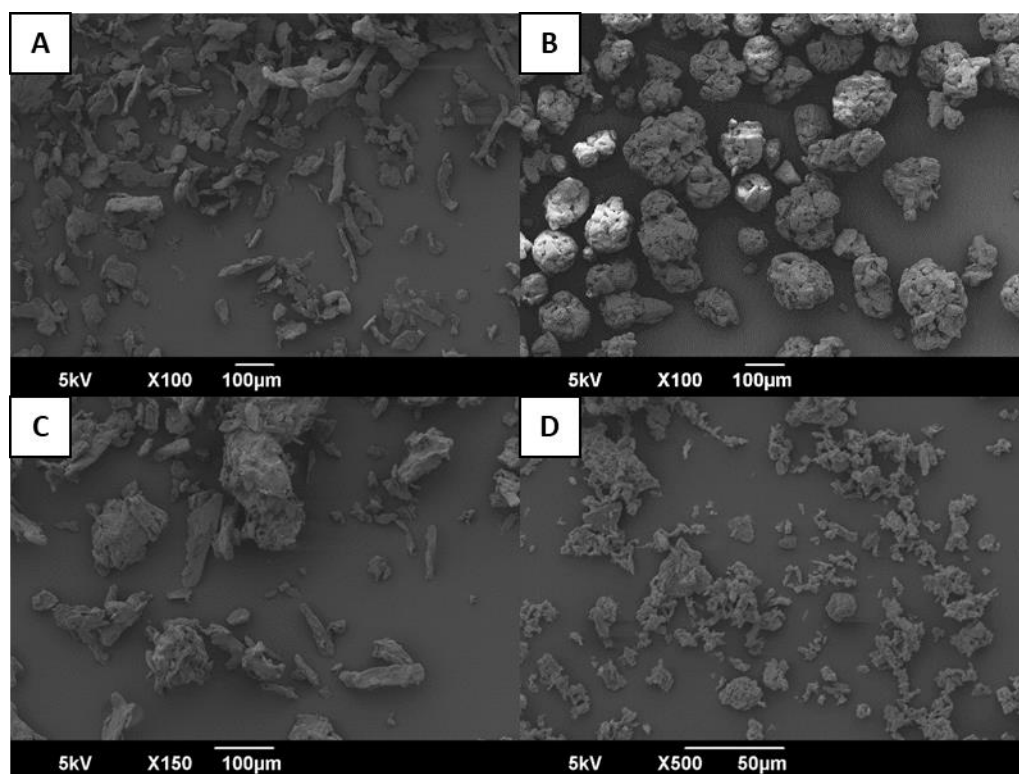


Figure 3.2. SEM micrographs of excipients in caffeine tablets: (A) HPMC at ×100 magnification, (B) lactose at ×100 magnification, (C) MCC at ×150 magnification and (D) MgSt at ×500 magnification.

3.4.2 Morphological characterisation and optical analysis of cross-sectioned caffeine tablets

3.4.2.1 Morphological characterisation of tablets using SEM

In addition to particle morphological characterisation, SEM was applied to the caffeine tablet to assess if this technique was able to differentiate the different components. According to the sample preparation method, as described in Section 3.3.2.2, the tablet was frozen in liquid nitrogen to avoid streak lines observed in the tablet which was not frozen. Figure 3.3A and B illustrate these streak lines (white arrows) by comparing the two sample preparation approaches. Without freezing, the compressed powder was more easily damaged by sectioning using the razor blade. On the other hand, freezing converts water in the tablet to ice, leading to the more rigid tablet. The firm tablet was gently cut in half using a razor blade to maintain the shape of tablet components as much as possible.

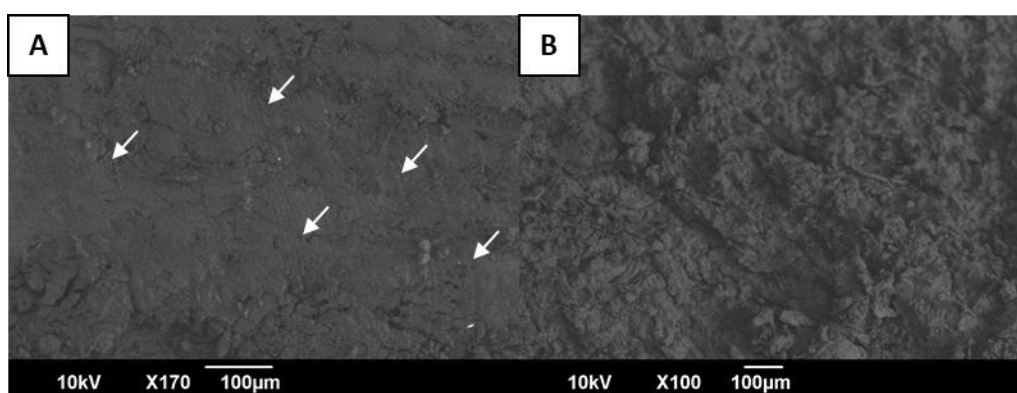


Figure 3.3. SEM micrographs of cross-sectioned caffeine tablets: (A) without liquid nitrogen freezing and (B) with liquid nitrogen freezing. White arrows highlight streak lines.

It can be seen from Figure 3.4 that features with different shapes and sizes were observed. Based on the SEM micrograph of raw materials (Figure 3.2), a long fibrous type of feature is likely to be HPMC or MCC which are cellulosic derivatives. In contrast, globular features are proposed to be either lactose or caffeine clusters.

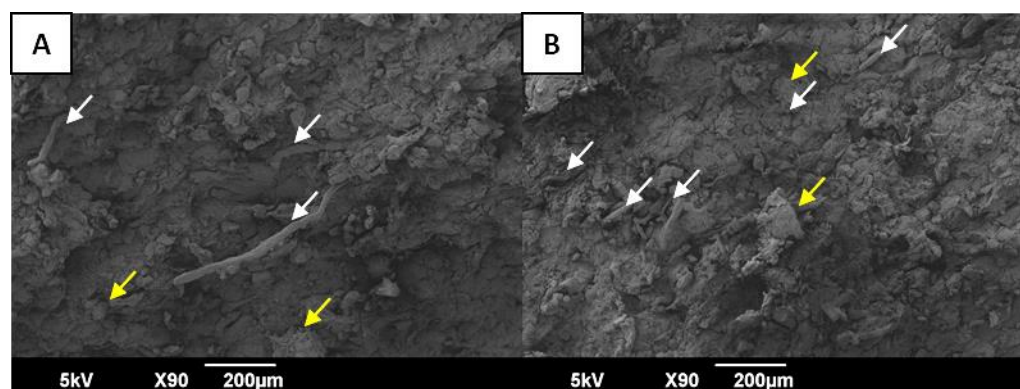


Figure 3.4. SEM micrograph of the half tablet in different regions using $\times 90$ magnification. White arrows highlight long fibrous features, whereas yellow arrows highlight globular features.

Despite observation of multiple ingredients using SEM can be made but a visual assessment can be subjective as it is difficult to distinguish similar features within the cross-section such as HPMC vs MCC particles and caffeine vs lactose particles. The identification of chemicals in the tablet using SEM may be substantiated by SEM-EDX. The EDX spectra contain peaks which correspond to all the varied elements presented in the sample leading to the identification of the elements. However, the active substances and excipients in the tablet systems most likely consist of C, H, O or N elements which may make it difficult to distinguish each component. Specifically, the raw materials in this study only contain these four elements.

The sample preparation approach as mentioned in Section 3.3.2.2 was specifically needed for the SEM analysis as actual particle morphology can be achieved. However, rough surface generated due to a razor blade is not applicable for further analyses such as Raman and ToF-SIMS chemical imaging which require consistently flat surface. Applying a physical force to the tablet for tablet sectioning using the razor blade may cause the tablet to become compacted.

3.4.2.2 Optical imaging of tablets using light microscope

Instead of using a razor blade for sample preparation as applied to the tablet sample for SEM analysis in Section 3.4.2.1, the tablet was cross-sectioned by

ultra-microtomy which produced a rather flat surface. Figure 3.5A shows the schematic representation of a cross-section demonstrating the analysed area and Figure 3.5B shows the actual cross-sectioned tablet embedded in an amount of resin on an SEM specimen stub. An optical microscope was performed to assess how the tablet components distributed across the analysed area and whether or not the compounds of interest were found. It can be observed from the optical images (Figure 3.5C and D) of the tablet that the bright oblong features are visible and aligned with the y-axis on the cross-sectional plane. With regard to the SEM data in Figure 3.2A and C, these particles were alike HPMC or MCC which are fibrous polymers. This finding suggests that HPMC and MCC may be recognised from other tablet's ingredients. Optical imaging with the $\times 20$ magnification, as shown in Figure 3.5D reveals that there were small globules which could possibly be caffeine clusters, lactose or compressed fibrous polymers. There were more opaque particles in the background which were not confidently identified. As such it was determined that chemical imaging may be required to chemically identify the components in the cross-sectioned tablet.

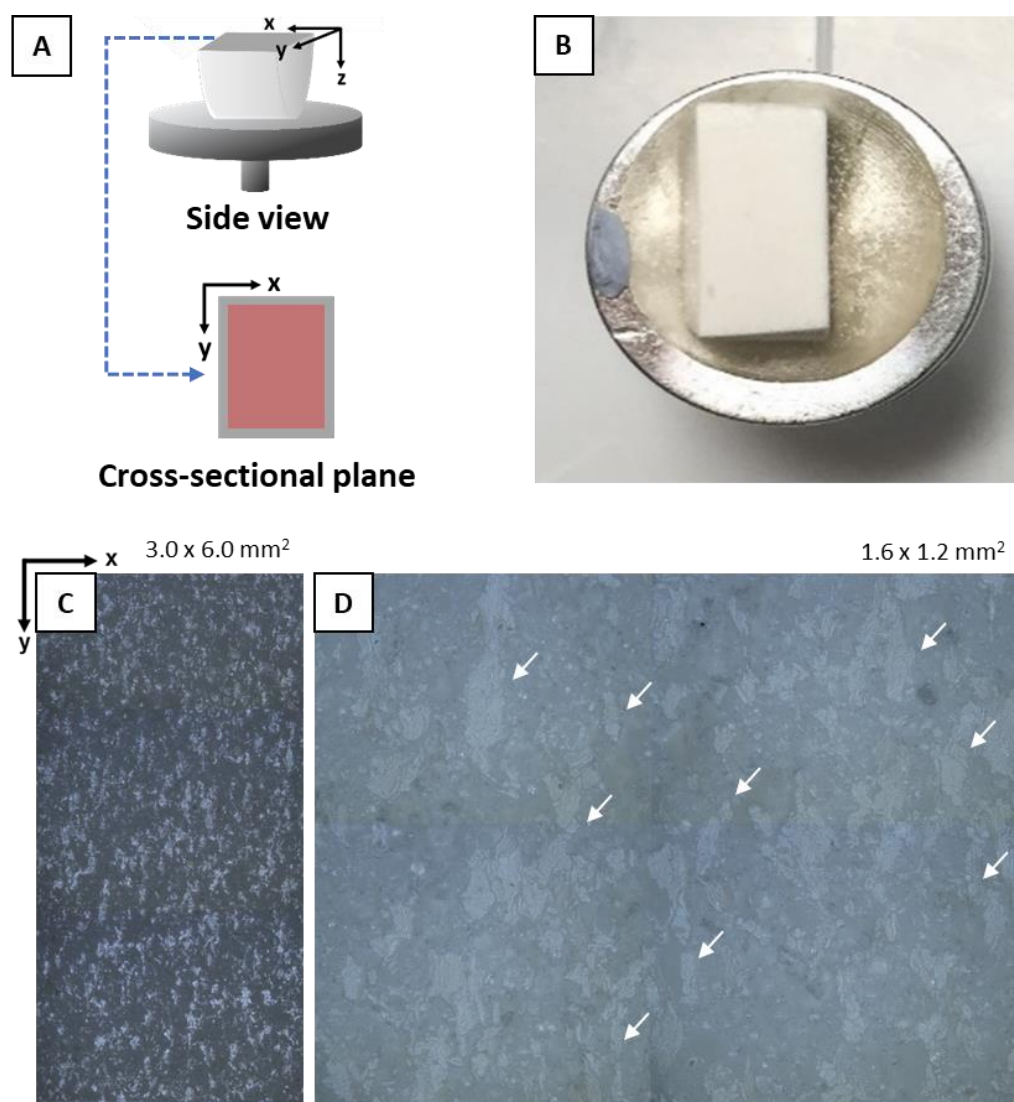


Figure 3.5. (A) Schematic representation of the cross-sectioned caffeine tablet sample with an analysed area highlighted in red, (B) actual cross-sectioned tablet on an SEM stub and (C) optical images of the cross-sectioned caffeine tablet sample with $\times 5$ magnification and (D) with $\times 20$ magnification. White arrows indicate fibrous-like particles.

3.4.3 Chemical characterisation of raw materials and tablets using CRM

3.4.3.1 Identification of the tablet's components using CRM

Prior to Raman analysis of the tablet, raw materials used in the tablet were analysed using Raman spectroscopy. Figure 3.6 shows the Raman spectra of raw materials in the tablets including caffeine, HPMC, lactose, MCC and MgSt.

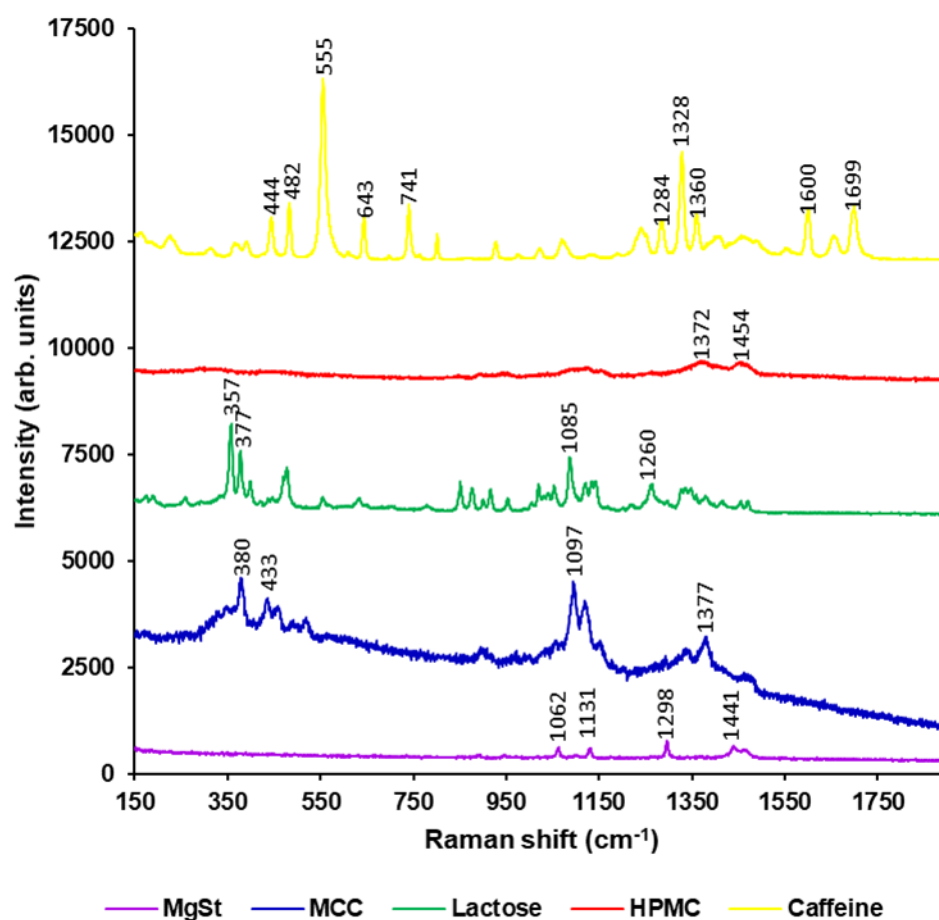


Figure 3.6. Raman spectra ($150 - 1900 \text{ cm}^{-1}$) of raw materials in the caffeine tablet.

The spectra of raw materials were compared to the data extracted by K-means cluster analysis (Section 2.2.7) which was applied to the analysis of tablets. The average spectra of components in the caffeine tablet are shown in Figure 3.7. There are four components identifiable using CRM in conjunction with K-means cluster analysis. MgSt was not detected, this may be expected to be as a result of the inability of the Raman technique to detect this substance at such a low concentration (0.5 %w/w). MgSt may also be less likely to be observed in the centre of a tablet due to its intended lubrication effect which would locate it towards the surface of the tablet. In theory, it is possible to observe the spectrum of any raw material when its particle size is above the step size. By contrast, when the particle size is smaller than the step size level, the material might be mixed with other compounds in a pixel. This then causes the observation of a mixed spectrum instead of the spectrum of a single component. In Figure 3.7,

the average Raman spectra of the tablet's components extracted by K-means cluster analysis were similar to those of the reference compounds (Figure 3.6) demonstrating that K-means cluster analysis can be used for identifying the tablet's components. Table 3.1 shows the Raman bands with their respective tentative assignments.

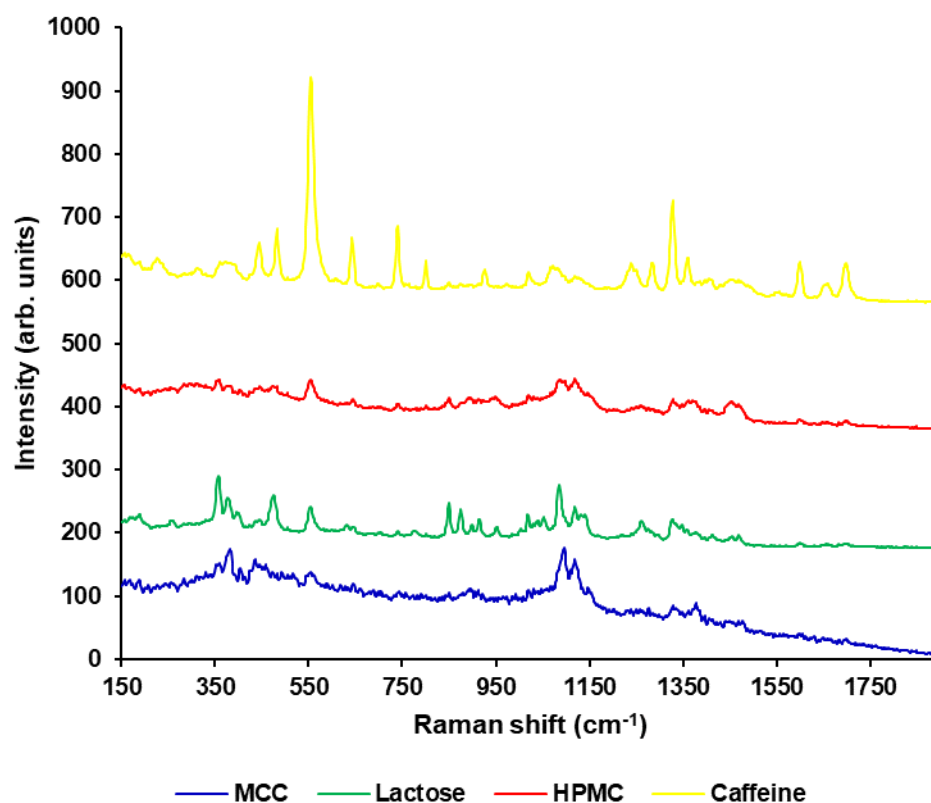


Figure 3.7. Average Raman spectra ($150 - 1900 \text{ cm}^{-1}$) of components in the 15% HPMC tablets.

Table 3.1. Interpretation of Raman spectra of the compounds in caffeine tablets.

Name of ingredients	Raman shift (cm ⁻¹)	Tentative peak assignment
Caffeine	444	$\delta_s(\text{CH}_3)^{241}$, $\rho(\text{CH}_3)^{241}$, $\delta(\text{pyrimidine ring})^{242}$, $\delta(\text{CNO})^{242}$, $\delta(\text{CH})^{242}$
	482	Imidazole ring def. ²⁴¹ , OCN def. ²⁴¹⁻²⁴² , $\delta(\text{CH})^{241}$
	555	$\delta(\text{CH})^{241}$, $\delta_s(\text{CH}_3)^{241}$, $\rho(\text{CH}_3)^{241}$
	643	Imidazole ring def. ²⁴¹ , OCN def. ²⁴¹
	741	$\delta_s(\text{CH}_3)^{241}$, $\rho(\text{CH}_3)^{241}$
	1284	$\nu_s(\text{CN})^{241}$, $\rho(\text{CH}_3)^{241}$, $\nu(\text{imidazole ring})^{241}$
	1328	$\nu(\text{imidazole trigonal ring})^{241}$
	1360	$\nu(\text{CN})^{241}$, $\delta_s(\text{CH}_3)^{241}$
	1600	$\nu_s(\text{CC})^{241}$, $\nu_s(\text{CN})^{241}$, $\delta_s(\text{CH}_3)^{241}$
	1699	$\nu(\text{CO})^{241}$
HPMC	1372	$\delta(\text{CH})^{243}$
	1454	$\delta(\text{CH})^{243}$
Lactose	357	$\tau(\text{OH})^{244}$
	377	$\delta(\text{CCO})^{244}$
	1085	$\nu(\text{CC})^{244}$
	1260	$\delta(\text{CH})^{245}$
MCC	380	$\delta(\text{CCC})^{246}$, $\delta(\text{CO})^{246}$, $\delta(\text{CCO})^{246}$, ring def. ²⁴⁶
	433	$\delta(\text{CCC})^{246}$, $\delta(\text{CCO})^{246}$, ring def. ²⁴⁶
	1097	Unsubstituted cellulose sugar ring def. ²⁴⁷
	1377	$\delta(\text{CH})^{247}$
MgSt	1062	$\nu_{as}(\text{CC})^{245}$
	1131	$\nu_s(\text{CC})^{245}$
	1298	$\delta(\text{CH}_2)^{245}$
	1441	$\delta(\text{CH}_2)^{245}$

ν , stretching; δ , bending; ρ , rocking; τ , twisting; s , symmetric; as , asymmetric; def., deformation

3.4.3.2 Characterisation of the caffeine tablet using CRM

In order to reconstruct Raman maps of four identifiable components in the caffeine tablets, chemometric algorithms were chosen and compared. The combined images based on basis analysis and K-means cluster analysis are shown in Figure 3.8B and C respectively. The outermost black areas of the combined images as shown in Figure 3.8B and C refer to the edge where there were no data. It can be seen in Figure 3.8B that sample alignment had a significant effect on the quality of the combined Raman map calculated by basis analysis. Technically, the spectra of the raw materials are required for the basis analysis algorithm. The imaging data near the edge of the tablet was increasingly darker than the area in the middle demonstrating that the bottom area might not be horizontally aligned with respect to the focal plane. The Raman map calculated based on the basis analysis can be affected by other variability sources in the sample such as moisture adsorption and interaction between components. Contrary to basis analysis, cluster analysis can extract average spectra making it practical when raw materials are not available and when the samples are unlevelled. In this study, cluster analysis was selected to avoid the impact of sample alignment.

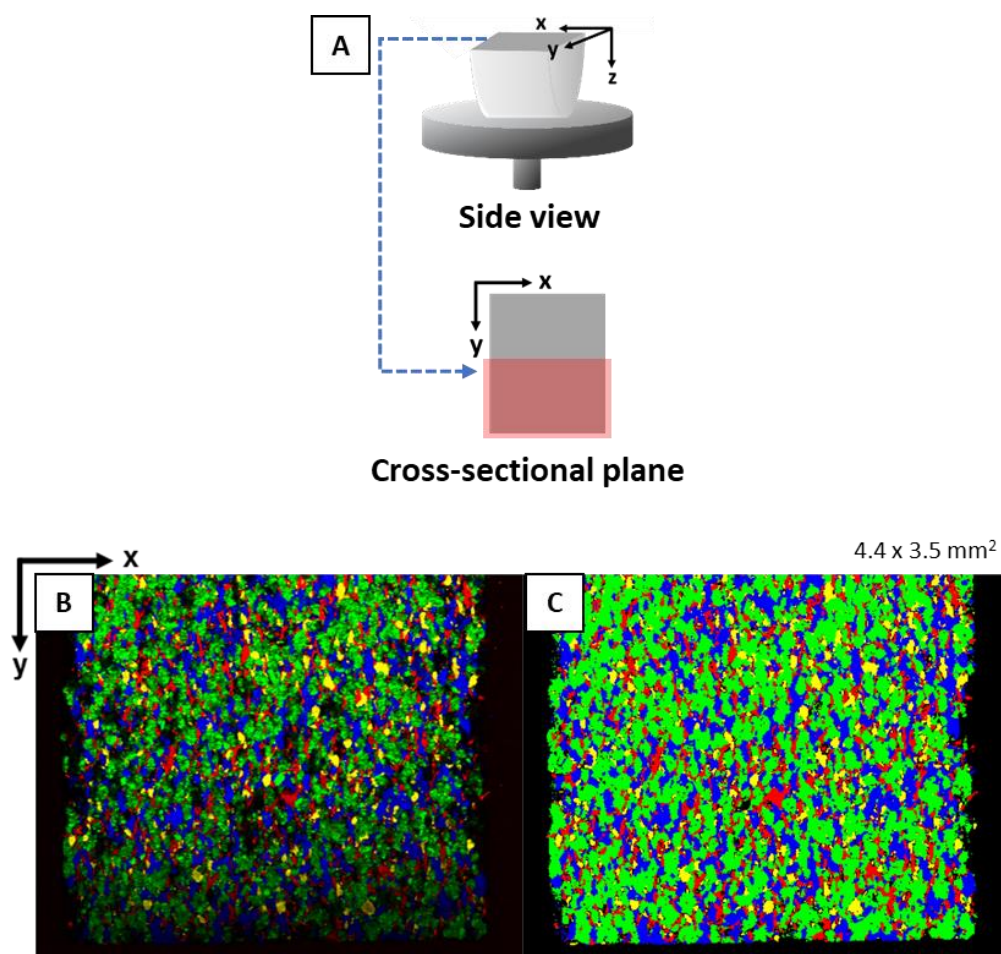


Figure 3.8. (A) Schematic representation of the cross-sectioned caffeine tablet sample with an analysed area highlighted in red and Raman images showing the comparison between (B) basis analysis and (C) K-means cluster analysis. Tablets contain caffeine (yellow), HPMC (red), lactose (green) and MCC (blue).

The Raman images of the components, including caffeine, HPMC, MCC and lactose, using K-means cluster analysis were reconstructed as presented in Figure 3.8C. Black spots within the cross-section refer to voids in the tablet. As stated in the introduction, the distribution of the components can be assessed using CRM. Caffeine clusters and particles (Figure 3.9A) were uniformly dispersed in the analysis area. It can be seen that the shape of the caffeine clusters and particles in the cross-sectioned tablet were similar to those observed in raw materials using SEM (Figure 3.1). Elongated HPMC particles (Figure 3.9B), interestingly, maintained their same shape as observed in the morphological analysis of raw material using SEM (Figure 3.2A). In contrast, the MCC raw material particles were long and fibrous (Figure 3.2C), the cross-section image

analysis shows that they became clustered presumably following compression as can be seen in Figure 3.9C. The reason for this may be that MCC has dry binding properties.²⁴⁸ The lactose morphology was changed on the tablet matrix (Figure 3.9D) compared to those from the SEM data (Figure 3.2B). The form of spray-dried lactose is spherical to improve flowability. However, when it was compressed, lactose seems to transform into large clusters. This demonstrates the ability of lactose to improve the compressibility or tabletability of the compression mix.

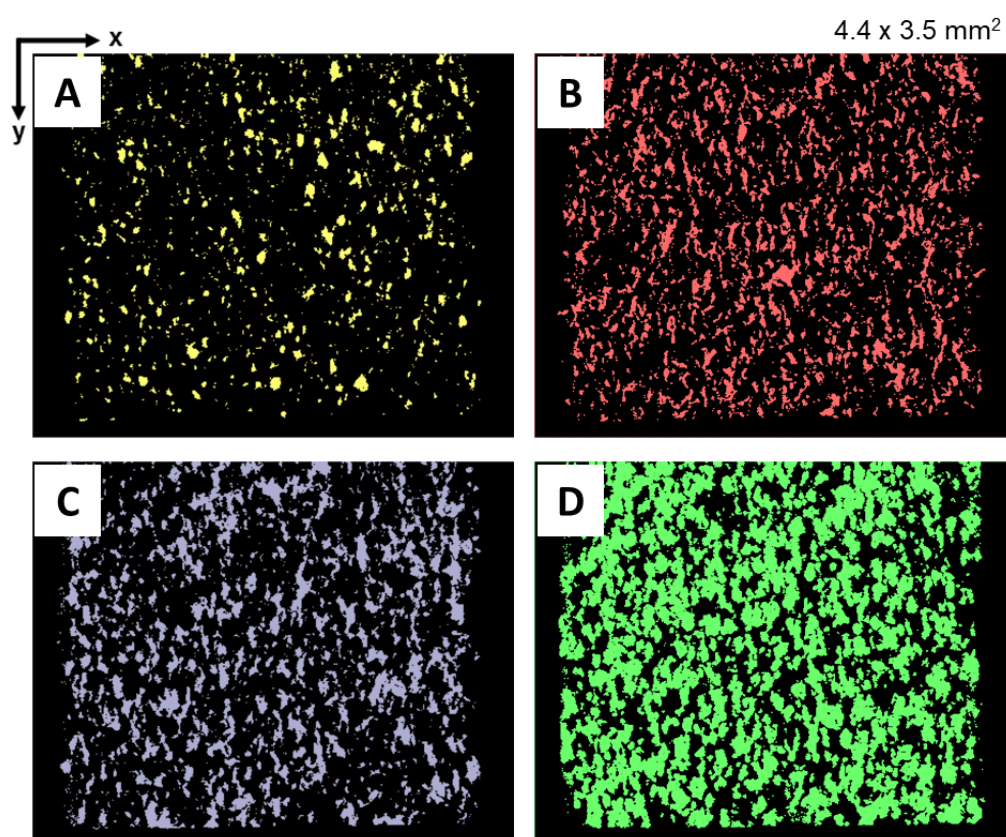


Figure 3.9. Raman images showing the distribution of components in the caffeine tablet: (A) caffeine, (B) HPMC, (C) MCC and (D) lactose, generated by K-means cluster analysis.

3.4.3.3 Comparison between Raman chemical imaging and optical imaging

It can be seen in Section 3.4.3.2 that Raman imaging facilitates the identification of the particles with similar characteristics. In this study, both HPMC and MCC were filamentous materials. As a result, it is difficult to utilise only an optical

imaging technique to confirm whether or not each particle is HPMC or MCC. Figure 3.10A shows the similarity in particle size and shape between HPMC and MCC particles when it is observed using light microscopy. The Raman image in Figure 3.10B confirms that the long fibrous particles could possibly be either HPMC or MCC. Furthermore, it is observed that in the matrix system these materials are less likely to absorb but are more likely to reflect the light resulting to becoming relatively bright particles in Figure 3.10A. Lactose (green colour in Figure 3.10B) and caffeine clusters (yellow colour in Figure 3.10B), on the other hand, seem to highly absorb the light which leads to the area with rather dark colour. It is interesting that light microscopy may distinguish HPMC and MCC particles from caffeine and lactose clusters due to difference in the properties for absorbing or reflecting the incident light.

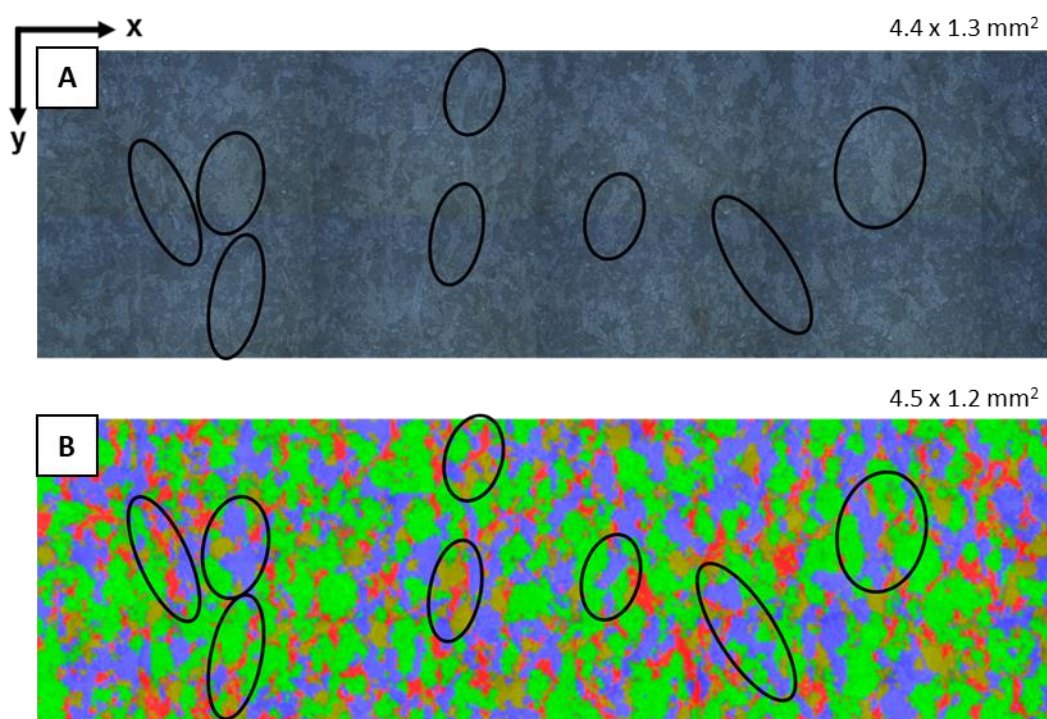


Figure 3.10. Comparison between (A) optical and (B) Raman imaging data of the 15 %w/w HPMC tablet containing caffeine (yellow), HPMC (red), lactose (green) and MCC (blue).

3.4.4 Chemical characterisation of raw materials and tablets using static ToF-SIMS

3.4.4.1 Identifying secondary ions of the caffeine tablet components

Reference samples of caffeine, HPMC, lactose, MCC and MgSt were analysed using static ToF-SIMS to establish the diagnostic secondary ions. The molecular structure for these reference materials can be found in Figure 2.1. This information was useful for the identification of the secondary ions. Figure 3.11 and Figure 3.12 show a range of peaks relating to caffeine, HPMC, lactose, MCC and MgSt in both polarities of the ToF-SIMS spectra. Criteria used to consider whether or not the ions were exclusive for the identification of the ingredients are specificity, selectivity and intensity. The ions must be specific to the corresponding compounds. Intact molecules, therefore, were favourable. The peaks of the ions should not be overlapped with other compounds in the tablet matrices. The ions with higher intensity were more preferable. In the ToF-SIMS positive spectra, it can be seen that the secondary ions of MCC (Figure 3.11D) were found in the mass spectra of other compounds. The examples for these include the $C_4H_9^+$ ion found in the caffeine, HPMC, lactose and MgSt spectra, the $C_4H_9O^+$ ion overlapped with the $C_3H_5O_2^+$ ion of the HPMC, lactose and MgSt spectra and the $C_6H_{11}O_5^+$ ion found in lactose. Furthermore, the secondary ions relating to MCC in the ToF-SIMS negative spectrum (Figure 3.12D) were mostly found in the negative spectrum of HPMC and lactose. Additionally, there were ions corresponding to MgSt (Figure 3.12E) that were presented in the ToF-SIMS negative spectrum of HPMC, lactose and MCC (Figure 3.12B-D). Owing to the fact that the negative polarity of ToF-SIMS spectra can identify fewer excipients, the positive ToF-SIMS mode was preferable to the negative one.

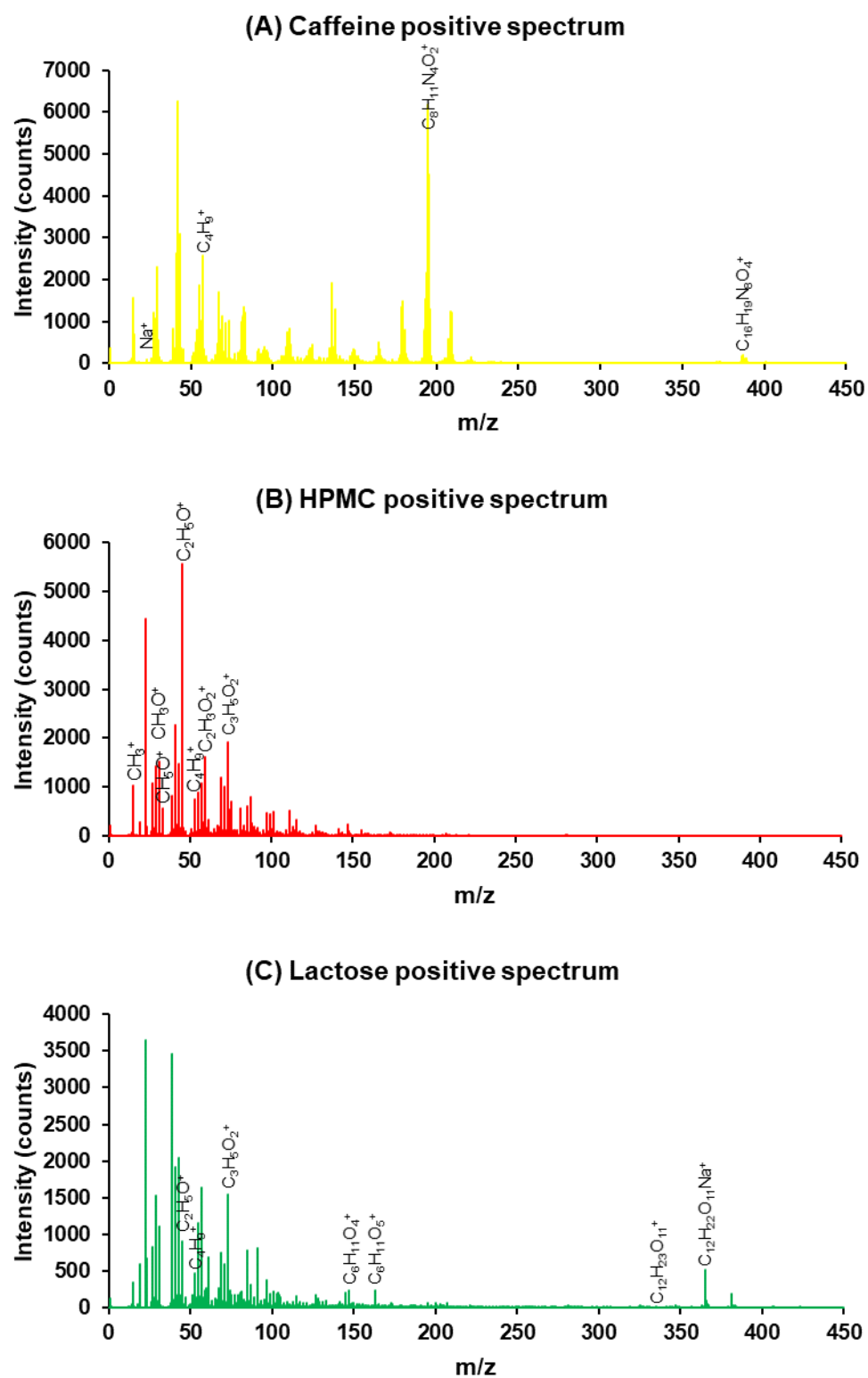


Figure 3.11. ToF-SIMS positive spectra of raw materials in the caffeine tablet: (A) caffeine, (B) HPMC, (C) lactose, (D) MCC and (E) MgSt.

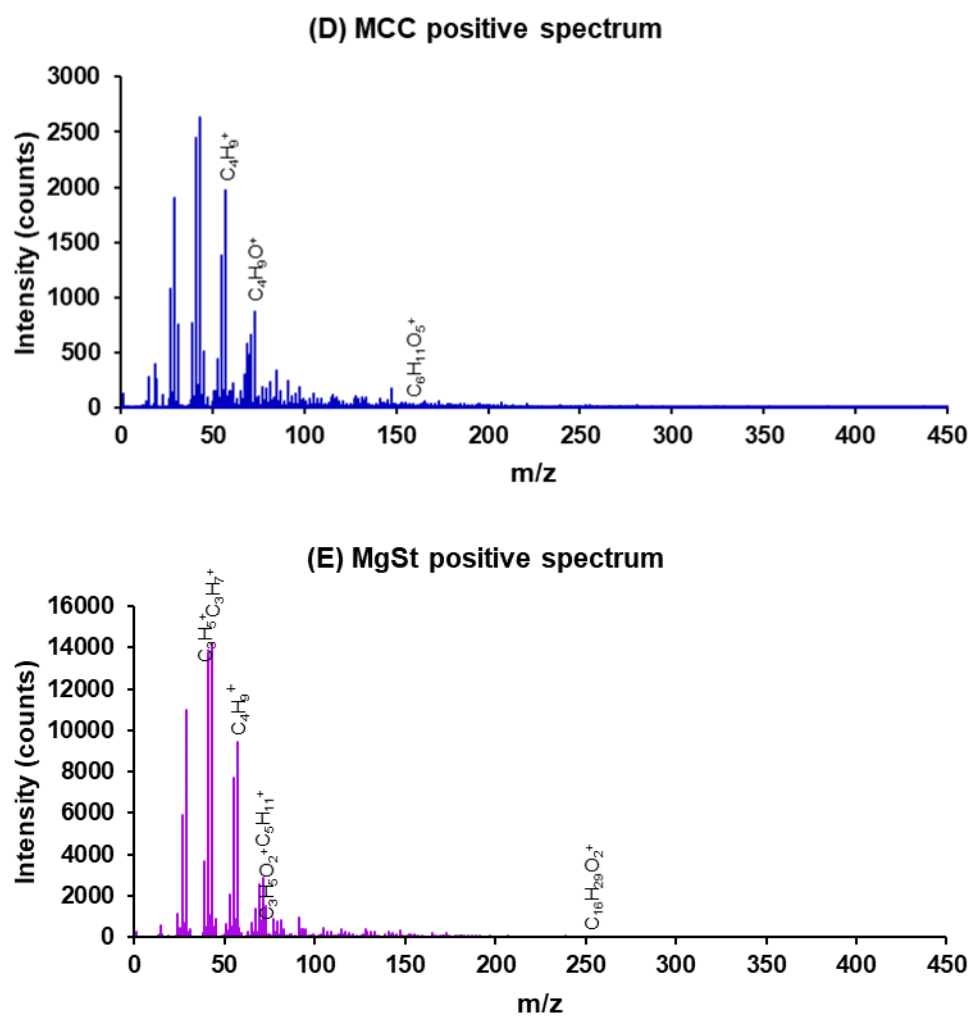


Figure 3.11 (continued). ToF-SIMS positive spectra of raw materials in the caffeine tablet: (A) caffeine, (B) HPMC, (C) lactose, (D) MCC and (E) MgSt.

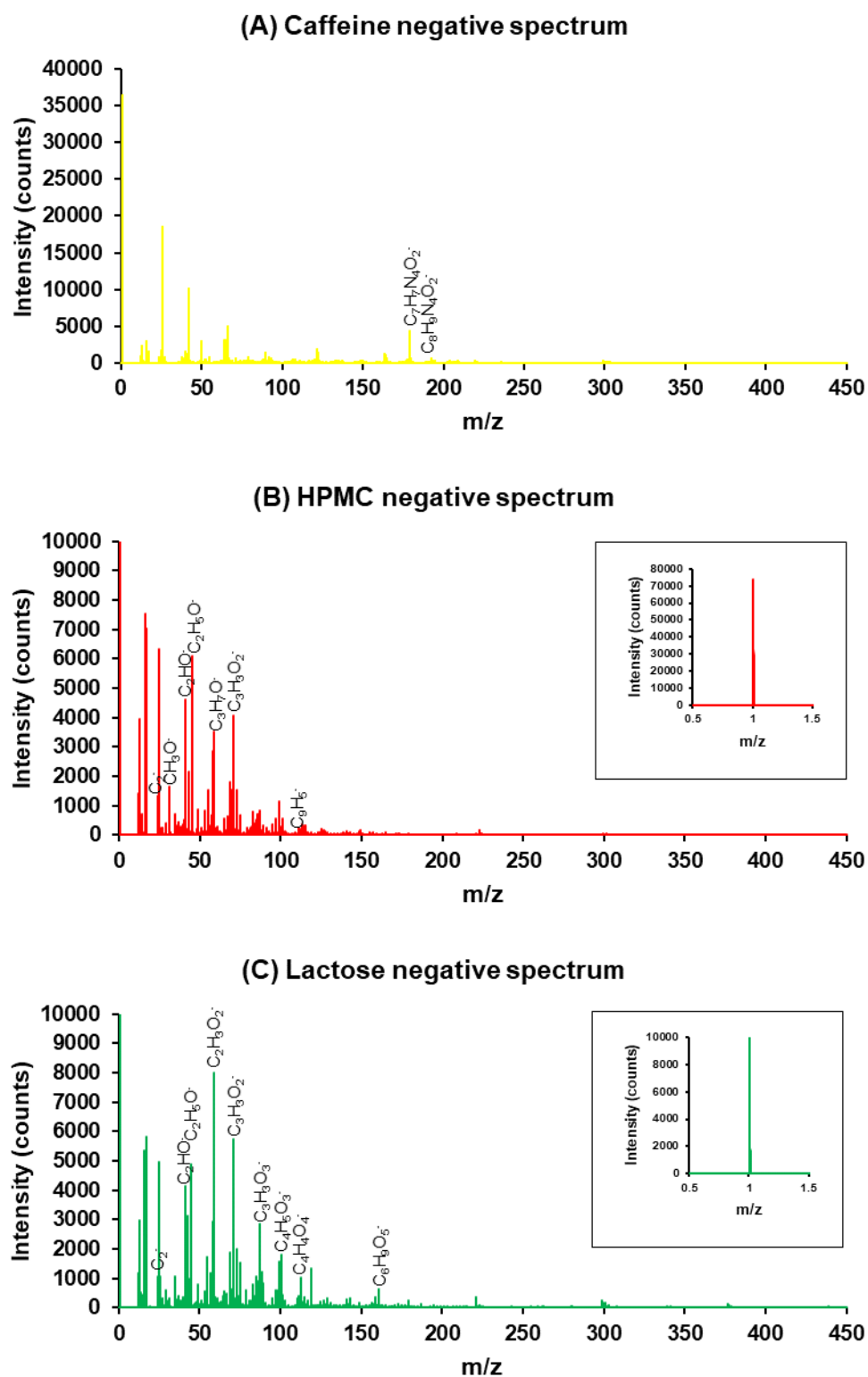


Figure 3.12. ToF-SIMS negative spectra of raw materials in the caffeine tablet: (A) caffeine, (B) HPMC, (C) lactose, (D) MCC and (E) MgSt.

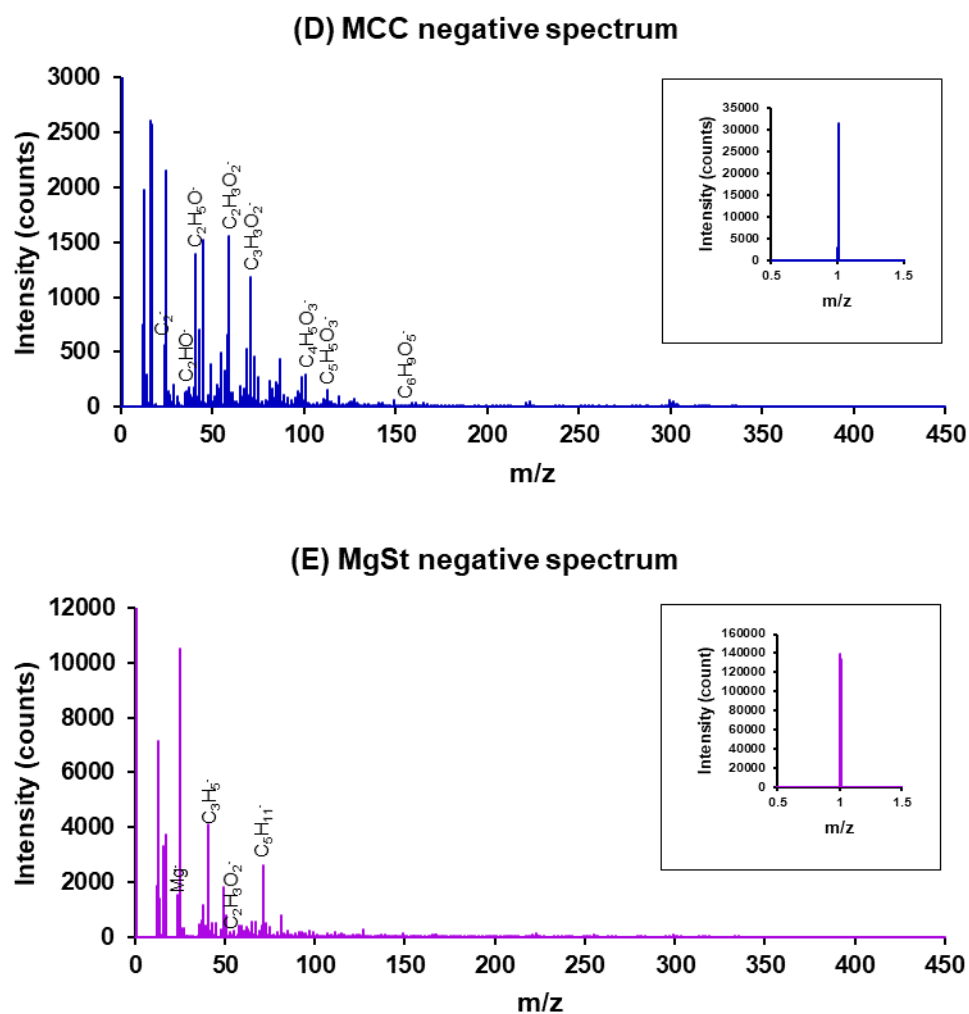


Figure 3.12 (continued). ToF-SIMS negative spectra of raw materials in the caffeine tablet: (A) caffeine, (B) HPMC, (C) lactose, (D) MCC and (E) MgSt.

A series of secondary ions including the intact molecules and fragments emitted from each reference compound were outlined in Table 3.2. The secondary ions highlighted in red colour were selected to be the diagnostic secondary ion of each raw material.

Table 3.2. Chemical information of the compounds in caffeine tablets including the chemical formula, molecular mass, characteristic peak, chemical composition and description.

Name of ingredients	Mass (m/z)	Chemical composition	Description
Caffeine $C_8H_{10}N_4O_2$ 194.1906 g/mol	22.98	Na^+	Na
	194.93	$C_8H_{11}N_4O_2^+$	Intact molecule + H
	386.79	$C_{16}H_{19}N_8O_4^+$	2 Intact molecules
	178.86	$C_7H_7N_4O_2^-$	Intact molecule - CH_3
	192.83	$C_8H_9N_4O_2^-$	Intact molecule - H
HPMC $C_{56}H_{108}O_{30}$ 1261.43872 g/mol	15.02	CH_3^+	Cellulose fragment
	31.03	CH_3O^+	Cellulose fragment
	33.04	CH_5O^+	Cellulose fragment
	45.05	$C_2H_5O^+$	Cellulose fragment
	59.07	$C_2H_3O_2^+$	Cellulose fragment
	31.02	CH_3O^-	Cellulose fragment
	45.00	$C_2H_5O^-$	Cellulose fragment
Lactose $C_{12}H_{22}O_{11}$ 342.29648 g/mol	59.01	$C_3H_7O^-$	Cellulose fragment
	45.04	$C_2H_5O^+$	Lactose fragment
	73.04	$C_3H_5O_2^+$	Lactose fragment
	147.19	$C_6H_{11}O_4^+$	One ring of the intact molecule - O
	163.13	$C_6H_{11}O_5^+$	One ring of the intact molecule
	343.26	$C_{12}H_{23}O_{11}^+$	Intact molecule + H
	365.23	$C_{12}H_{22}O_{11}Na^+$	Intact molecule + Na
	44.99	$C_2H_5O^-$	Lactose fragment
	59.00	$C_2H_3O_2^-$	Lactose fragment
	71.00	$C_3H_3O_2^-$	Lactose fragment
	86.99	$C_3H_3O_3^-$	Lactose fragment

Name of ingredients	Mass (m/z)	Chemical composition	Description
MCC C ₁₄ H ₂₆ O ₁₁ 370.34964 g/mol	57.12	C ₄ H ₉ ⁺	Cellulose fragment
	73.09	C ₄ H ₉ O ⁺	Cellulose fragment
	163.15	C ₆ H ₁₁ O ₅ ⁺	Intact molecule + H
	45.00	C ₂ H ₅ O ⁻	Cellulose fragment
	59.01	C ₂ H ₃ O ₂ ⁻	Cellulose fragment
	71.03	C ₃ H ₃ O ₂ ⁻	Cellulose fragment
	101.04	C ₄ H ₅ O ₃ ⁻	Cellulose fragment
	113.04	C ₅ H ₅ O ₃ ⁻	Cellulose fragment
	161.10	C ₆ H ₉ O ₅ ⁻	Cellulose fragment
Magnesium stearate C ₃₆ H ₇₀ MgO ₄ or Mg(C ₁₈ H ₃₅ O ₂) ₂ 591.2436 g/mol	23.99	Mg ⁺	Mg
	41.04	C ₃ H ₅ ⁺	Stearate fragment
	43.06	C ₃ H ₇ ⁺	Stearate fragment
	71.10	C ₅ H ₁₁ ⁺	Stearate fragment
	253.22	C ₁₆ H ₂₉ O ₂ ⁺	Stearate fragment
	24.00	Mg ⁻	Mg
	41.02	C ₃ H ₅ ⁻	Stearate fragment
	71.03	C ₅ H ₁₁ ⁻	Stearate fragment

As mentioned before, the MCC peaks overlapped with those from other compounds both in the positive (Figure 3.11) and negative (Figure 3.12) polarities. As a result, it is unable to identify exclusive secondary ions which represent MCC. However, due to the fact that the study focuses on the distribution of HPMC and caffeine in order to correlate the role of HPMC and caffeine in the formulation with their chemical distribution. MCC is not a primary interest so that this should be acceptable.

In the identification of diagnostic ions, the intact molecular ions are preferable due to their high chemical specificity. However, it may be not suitable if these ions are not exclusive to one material. As shown in Table 3.2, caffeine and lactose intact molecules were produced by the analysis of the reference materials. The intact molecular ion of caffeine, C₈H₁₁N₄O₂⁺, was highly specific

to caffeine with a high ion intensity of 9.21×10^5 counts. In the positive polarity lactose spectrum, cationised molecular ions can be observed at both m/z 343 and m/z 365 correspondings to the intact molecule plus H and the intact molecule plus Na respectively. Nonetheless, the $C_{12}H_{22}O_{11}Na^+$ molecular ion intensity was 8.40×10^4 counts which is 70 times higher than the $C_{12}H_{23}O_{11}^+$ molecular ion. An intact molecule of HPMC could not be identified and a large number of secondary ions obtained from the HPMC reference sample, such as $C_2H_5O^+$ and $C_2H_3O_2^+$ ions, overlapped with ions at the same m/z from other compounds. The CH_5O^+ peak is the only ion that was not found in other materials' mass spectra. Aside from good selectivity, it is representative of HPMC. Although it is accepted that this ion is a less specific chemical species, it is likely to arise from the methoxyl or hydroxypropoxyl substitutions of HPMC. Since the Mg^+ ion is specific to MgSt, it was selected to be the marker illustrating MgSt. The secondary ions highlighted with red colour in Table 3.2 were the finalised characteristic ions to be applied in the further analysis of caffeine tablets.

3.4.4.2 Component distribution the caffeine tablet using static ToF-SIMS

Based on the mass spectral information as mentioned in Section 3.4.4.1, a series of diagnostic secondary ions representing the distribution of caffeine, HPMC, lactose and MgSt taken from a cross-sectioned 15 %w/w HPMC tablet were reconstructed and overlaid (Figure 3.13). The area in red in Figure 3.13A indicates the representative area of ToF-SIMS analysis. From this it can be observed that the distributional homogeneity of caffeine and excipients in the tablet can be evaluated. The homogeneous distribution of clumpy caffeine clusters was found. HPMC particles were uniformly distributed and they remained the same as shown in the SEM data (Figure 3.2A) suggesting that the powder mixture was blended adequately. In addition to findings from Raman analysis, it was able to identify a few spots of MgSt using ToF-SIMS. However, MCC was not identified due to its secondary ions being overlapped with other components as mentioned in a previous section (Section 3.4.4.1).

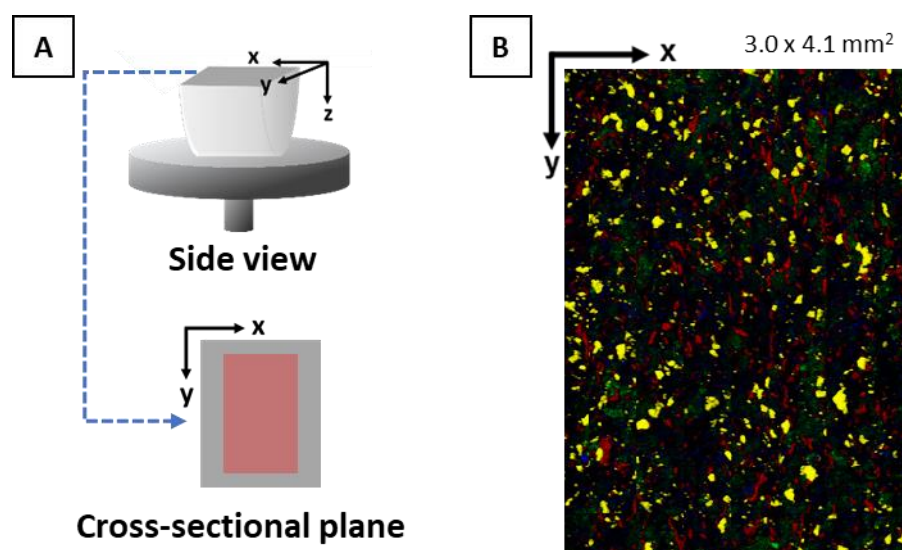


Figure 3.13. (A) Schematic representation of the cross-sectioned caffeine tablet sample with an analysed area highlighted in red and (B) ToF-SIMS overlay of caffeine (yellow), HPMC (red), lactose (green) and MgSt (blue).

Individual secondary ion images of the components in the cross-sectioned 15 %w/w HPMC tablet are presented in Figure 3.14. The $C_8H_{11}N_4O_2^+$ (Figure 3.14B) and CH_5O^+ (Figure 3.14C) images demonstrate the separate distribution of caffeine clusters and HPMC particles respectively. However, it can be assumed that the distribution of HPMC may be different when the concentration or particle size of HPMC are changed. While representative ions of caffeine and HPMC shows individual separation of their corresponding chemicals, the ToF-SIMS image of $C_{12}H_{22}O_{11}Na^+$ ion (Figure 3.14E) suggests that lactose particles became agglomerated to form clusters. Agglomerated lactose may be attributed to its very good compaction property. Agglomeration of lactose is expected due to the function of lactose for being a diluent in the formulation. Interestingly, Mg^+ ions were tremendously identified as a continuous film using ToF-SIMS. However, this could be suspected that the Mg^+ ion may not be sufficiently selective to MgSt. This may be attributed by overlapping of ions such as C_2^+ , which possess a molar mass of 24.02, in other ingredients. This result also supplements Raman imaging (Section 3.4.3.2) which could not find a negligible amount of MgSt in the formulation.

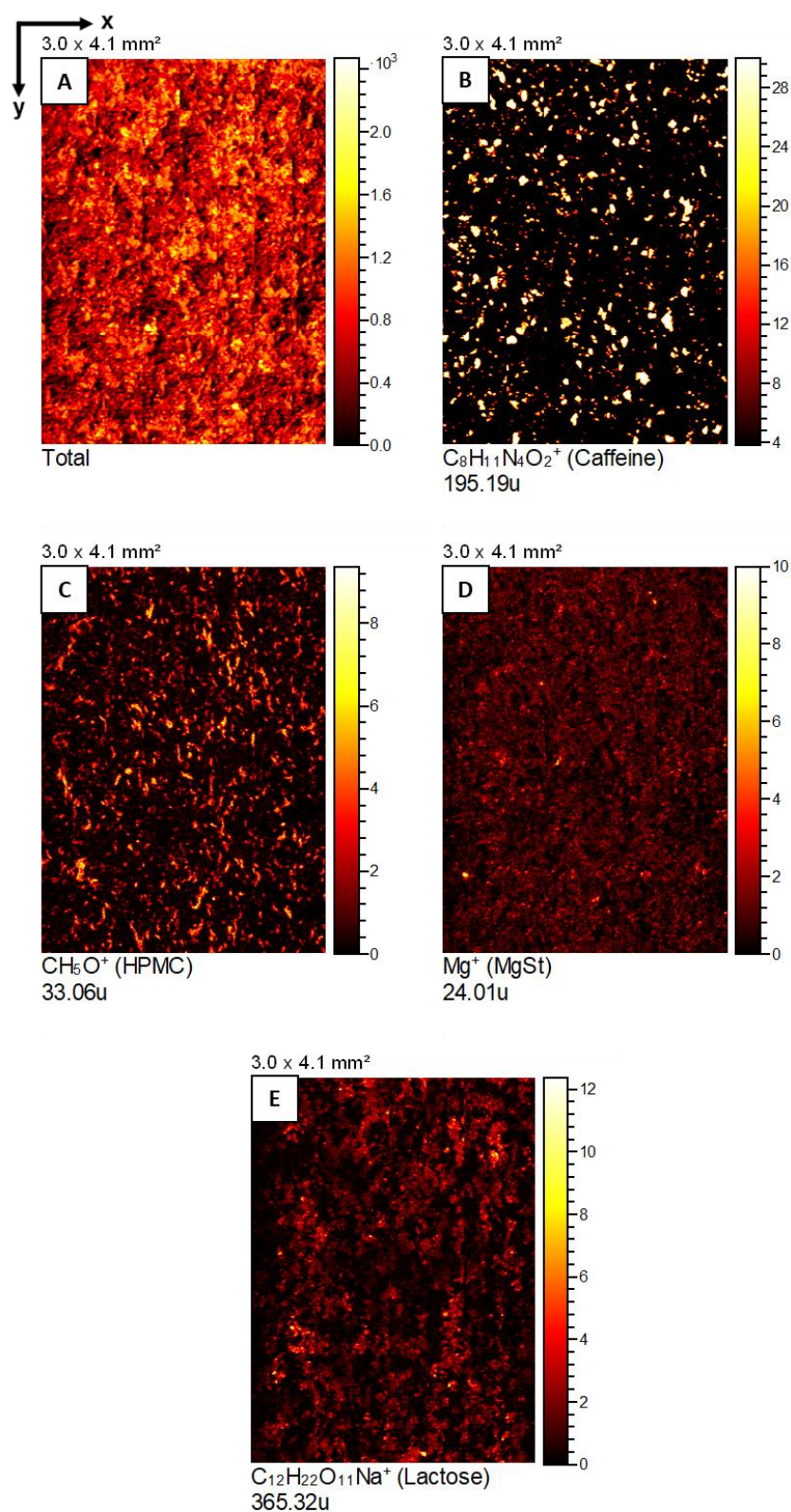


Figure 3.14. ToF-SIMS images of a cross-sectioned caffeine tablet sample containing 15%w/w of HPMC with <125 μm of HPMC particle size fraction representing (A) total ions, (B) caffeine, (C) HPMC, (D) MgSt and (E) lactose.

3.5 Conclusions

The main goal of Chapter 3 is to employ the combination of characterisation techniques in material sciences, ranging from optical microscopy, electron microscopy, vibrational spectroscopy to secondary ion mass spectrometry, with purposes of characterising the raw materials and determining the spatial distribution of key components in pharmaceutical tablets.

The hydrophilic matrix tablet with caffeine as an active substance and HPMC 2208 as a hydrophilic polymer is the main focus of this study. Prior to tablet analysis, the morphology of caffeine and the excipients was characterised using SEM. The characterisation of raw materials using SEM, CRM and ToF-SIMS aid in the identification of ingredients in the tablet. The sample preparation for the tablet system is needed to gain access to the internal structure of the tablet as well as to prepare the flat surface for further chemical analysis. It was found from optical data that it is visually difficult to differentiate HPMC from MCC due to their similar morphologies in terms of shape and size. Chemical imaging approaches including CRM and ToF-SIMS can provide spatially-resolved chemical information of pharmaceutical tablets. The characteristic of the tablet's components in the matrix tablet was thoroughly examined. It was found that the distributional homogeneity of caffeine and HPMC in the tablet formulation was quite uniform suggesting that there was sufficient mixing time during blending. The results have also led to understanding the behaviour and function of ingredients such as MCC and lactose in the formulation.

Chapter 4:

Relationship between tablet microstructure and dissolution performance of caffeine from hydrophilic matrix tablets

4.1 Introduction

It has been widely demonstrated that hydroxypropyl methylcellulose (HPMC) impacts on the release mechanism of an active substance. There have been a number of HPMC-relating factors controlling drug dissolution from hydrophilic matrices (Section 1.2.5.2). Such factors include HPMC content and particle size. The impact of HPMC factors can be supported by the idea of percolation theory. The percolation threshold is practically defined as the minimum level of HPMC in a formulation where increasing HPMC amount does not significantly reduce a dissolution rate. The formation of polymer particle connectivity may possibly be formed at the percolation threshold. As a consequence, the level of HPMC particles may dominantly control the overall release performance of the dosage form.

In vitro drug release testing is crucially important to assess the performance of tablets and needed to submit to the Food and Drug Administration (FDA) for new drug applications (NDAs). The findings from dissolution apparatuses can be obtained by collecting from solutions. Therefore, chemical information does not directly come from the solid form of tablets. The findings from the dissolution tests are not intrinsically spatially-resolved. As a result, dissolution tests cannot provide evidence to the link between tablet formulation factors and drug dissolution. Chemical imaging of tablets reveals valuable information, as shown in Chapter 3. Utilising CRM and ToF-SIMS would elucidate a material-structure-function relationship in tablets.

4.2 Aim and objectives

The aim of this chapter is to understand the relationship between tablet microstructure and drug release from hydrophilic matrix tablets. The objectives are:

- (1) Visualise the distribution of major components in the internal structure of tablets using CRM and ToF-SIMS.
- (2) Evaluate the influence of polymer concentrations and particle size fractions on the microstructure of tablets.
- (3) Correlate HPMC parameters obtained using ToF-SIMS with the dissolution profiles of caffeine from hydrophilic matrices.
- (4) Develop mathematical models based on critical HPMC parameters for predicting the cumulative dissolution of caffeine at different time points.

4.3 Materials and methods

4.3.1 Materials

Raw materials include caffeine (Sigma-Aldrich, UK), hypromellose 2208 USP (Methocel K4M Premium CR grade) (Colorcon, UK) as abbreviated as HPMC, lactose (316 NF Fast Flo, Foremost Farms, USA) (MSD, UK), Microcrystalline cellulose, as abbreviated as MCC (Avicel PH 102, FMC Biopolymer) (MSD, UK), and magnesium stearate (MgSt) (Sigma-Aldrich, UK).

4.3.2 Tablet formulation

Caffeine tablet formulations in this study can be divided into two series including a polymer concentration and a particle size series. For the concentration series, the content of HPMC in the tablet was varied from 7.5 to 30%w/w with a fixed particle size fraction of less than 125 μm . On the contrary, 15%w/w HPMC remained constant in the particle size series with variation in particle size fractions from below 45 μm to above 125 μm . The formulation and manufacture of tablet formulations can be found in Section 2.2.1.

4.3.3 Preparation of cross-sectioned tablets

A third of the tablets was initially removed using a razor blade on one radial side. The cut surface was then fixed onto an aluminium stub using Vitralit (Panacol, Germany), and it was subsequently exposed to blue light for approximately 5 minutes to induce polymerisation. Araldite Rapid (Huntsman Advanced Materials, Switzerland) is an alternative adhesive. Nonetheless, a polymerisation of Araldite Rapid may be for about 12 hours at room temperature to be fully complete as it requires plenty amount of the embedding compound for this size of the sample. Sufficient amount of resin for mounting is a key to ensure that the ToF-SIMS's edge effect (Section 2.2.9.6) is diminished. The tablet sample needs to embed in and surrounded by a sufficient amount of resin. Following this step, the tablet was automatically cross-sectioned with a PowerTome XL ultramicrotome (Boeckeler Instruments, US) using an 8 mm diamond knife

(RMC diamond knives, the United States). Subsequently, the tablet's surface was flat, smooth and levelled.

4.3.4 Component distribution using CRM

Raman analysis was performed using an alpha 300 WITec confocal Raman system (WITec Inc., Ulm, Germany). The samples were illuminated by a 785 nm near-Infrared laser source. This source was selected due to reduced fluorescence of MCC compared to other lasers. An objective of $\times 20$ magnification with 0.4 of numerical aperture (Zeiss, UK) was used for both optical image stitching and Raman mapping. The Raman spectra were taken with a spectral range of $100 - 2000 \text{ cm}^{-1}$. A spectrum was collected per pixel with an acquisition time of 0.3327 s. With a step size of $10 \text{ }\mu\text{m}$, Raman mapping ($n = 3$) was collected in 300 points per line and 350 lines per image, thereby collecting a final $3.0 \times 3.5 \text{ mm}^2$ image size.

WITec Project FOUR software (WITec Inc., Ulm, Germany) was used to analyse Raman hyperspectral data. Spectral pre-processing which includes cosmic ray removal and background subtraction was performed to minimise the background noise. K-means cluster analysis was used to identify similar spectra and classify the multispectral data into a number of clusters.

4.3.5 Component distribution using ToF-SIMS

ToF-SIMS spectra and imaging acquisitions in this study were performed using a ToF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany). Reference samples of caffeine, HPMC, lactose, MCC and MgSt were acquired to identify characteristic secondary ions. The preparation of powder samples is aforementioned in 3.3.2.1. The tablet samples ($n = 3$) were analysed using a 25 keV Bi_3^+ primary ion source, rastered over a $3.0 \times 4.1 \text{ mm}^2$ area. The total primary ion beam dose for each analysed area was kept below $1 \times 10^{12} \text{ ions/cm}^2$, thereby ensuring static conditions. The analysis area was located centrally to the sample cross-sectioned plane. In order to compensate any surface charge, an electron flood gun was used to provide low-energy electrons (20 eV). As shown previously in Section 3.4.4 that more unique secondary ions could be exhibited

in positive polarity mode than the negative one, data acquisition was then in positive polarity. Data processing was subsequently acquired using SurfaceLab 6 (ION-TOF GmbH, Münster, Germany).

4.3.6 Cumulative release test of caffeine from matrix tablets

The dissolution tests (USP II apparatus) were carried out in a volume of 900 mL of de-gassed, de-ionized water at a paddle speed of 50 RPM and 37 ± 0.5 °C. Tablets were held in a sinker to prevent floating or adhesion to the dissolution vessel wall. Automated sampling and absorbance at a wavelength of 273 nm were taken at different time points (at 4, 6, 8, 10, 15, 30, 45 and 60 minutes) in 10 mm quartz flow-through cells, using an 8453 Agilent UV/Vis spectrophotometer system equipped with Chemstation software version 08(03) (Agilent, USA).

4.3.7 Image analysis

ImageJ 1.52a software (National Institutes of Health, USA) was performed to assess the uniformity of HPMC particles and particle characteristics. A resolution of 0.212 and 0.250 μm per pixel was set for binary images converting from Raman and ToF-SIMS images respectively. Only the HPMC mapping was used to establish a concept of dissolution correlation. The step to extracting HPMC parameters from the chemical images can be seen in Figure 4.1. The colour threshold was adjusted to justify the boundary of each HPMC particle. Then, chemical maps of HPMC particles were converted into binary images demonstrating HPMC in white colour (colour value of 255). A median filter was applied for the purpose of removing noises using the ‘Despeckle’ command. This step also improved the edge detection of the particles. The median filter works by replacing each pixel with the median value in its 3×3 neighbourhood. Skeletonised images were developed in order to determine the connectivity of the HPMC particles. The free ImageJ plugin called ‘Skeleton Intersections’ was used to identify the locations of the intersections of HPMC skeletons and calculate a number of the intersections. The plugin is available online at http://jvsmicroscope.uta.fi/?q=skeleton_intersections. The mean inter-particle

distance, which is the average distance between every pair of the nearest neighbour particles, was calculated. This was contributed by the development of specific plugin ‘Graph’, which is freely available from the internet at <https://imagej.nih.gov/ij/plugins/graph/index.html>. Other image parameters including particle count, particle perimeter, particle area and Feret angle, were obtained using the ‘Analyze Particles’ command. For Feret angle data, the histogram with the bin centre of 10 was produced to determine the frequency distribution. The histogram of Feret angle values was fitted with Gaussian distributions.

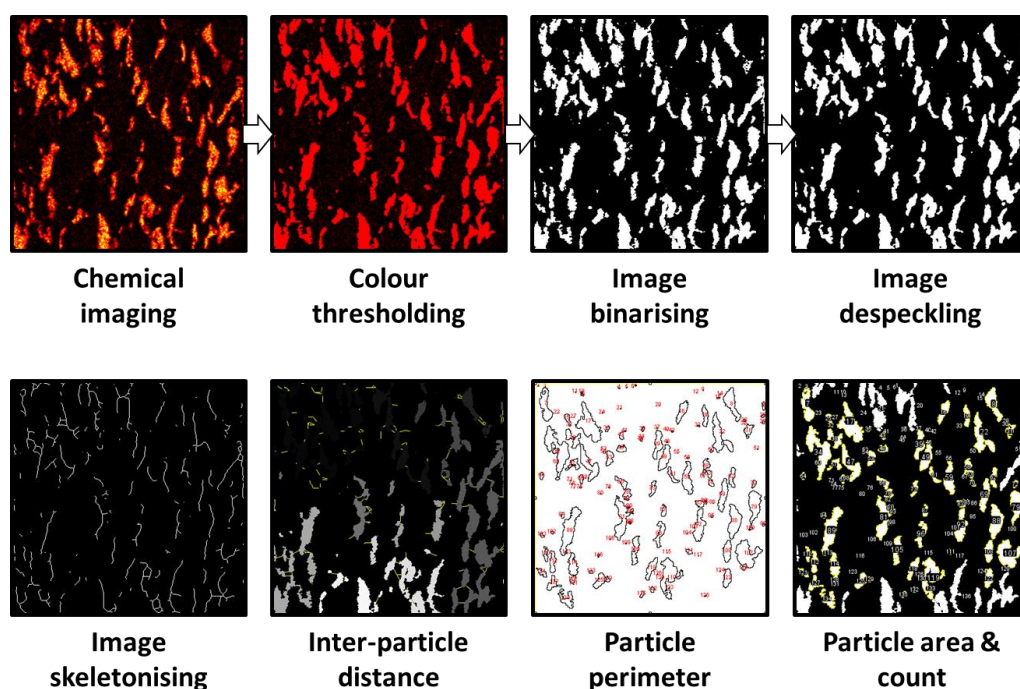


Figure 4.1. Schematic representation showing the steps of image processing and parameters extracted from ToF-SIMS images for quantitative analysis of HPMC parameters using ImageJ and its plugins.

4.4 Results and discussion

4.4.1 Component distribution of cross-sectioned tablets using CRM: effect of polymer concentration

The correlation between the effects of formulation factors and drug release has been presented in Chapter 1, from Section 1.2.5.1 to 1.2.5.3. However, the rationale behind this relationship has been significantly overlooked. It is hypothesised that investigating the microstructure and chemical distribution of key tablet components may enable an understanding of this relationship in hydrophilic matrices. The chemical images of hydrophilic matrices may also allow the determination of the percolation threshold where the connectivity and uniformity of HPMC particles formed. Raman has been used widely in the application of compositional imaging as mentioned in Section 1.3.3.4. The HPMC content is one of the critical formulation factors impacting the performance of HPMC as it may affect the formation of gel barriers. Tablets with differing HPMC content were examined by Raman analysis to elucidate the chemical microstructure of a cross-sectioned tablet surface.

Figure 4.2 shows the Raman imaging data of 7.5 and 30 %w/w HPMC tablets where all detected components, caffeine, HPMC, lactose and MCC, were overlaid into one chemical map. The findings show that caffeine (displayed in yellow) was surrounded by the uniform mixture of HPMC and other diluents which is in agreement with the concept of hydrophilic matrices. The most interesting finding is that there were different characteristics of HPMC particles in each formulation. White arrows highlight the different shapes of HPMC particles as a function of HPMC content. In Figure 4.2A, HPMC particles were individually separate and distinct. Part of the particles was elongated in shape which is similar to HPMC raw materials observed in the SEM images (Figure 3.2, Section 3.4.1). On the other hand, a continuous network of mutually attached HPMC particles, indicated with white arrows in Figure 4.2B, can be clearly seen in the 30 %w/w. In term of tablet composition, lactose is relatively abundant compared to other components in both tablets as might be expected owing to it

being the highest composition in these formulations. As might be anticipated, the area of lactose and MCC particles decreased when increasing the amount of HPMC due to their being substituted by one another. The black area was devoid of data which denotes any void space in the tablets.

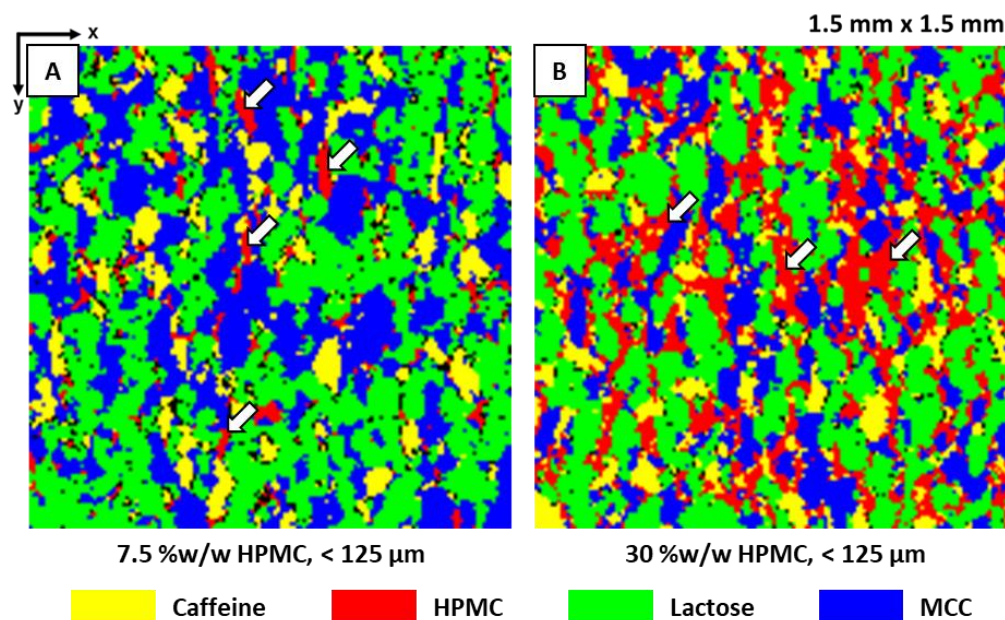


Figure 4.2. Representative Raman maps showing the comparison between the cross-sectioned tablets containing (A) 7.5 and (B) 30 %w/w HPMC with <125 μm of HPMC particle size fraction, representing caffeine (yellow), HPMC (red), lactose (green) and MCC (blue). White arrows highlight different characteristics of HPMC. Data taken from the tablets with the diameter of 8 mm and thickness of 4 mm.

In Figure 4.3, the Raman images of cross-sectioned caffeine tablets containing 7.5 – 30 %w/w HPMC (Figure 4.3A-F) demonstrate the individual distributions of HPMC (Figure 4.3I), caffeine (Figure 4.3II), lactose (Figure 4.3III) and MCC (Figure 4.3IV), generated by K-means cluster analysis. The uniform distribution of each component can be observed in all formulations, illustrating that the current mixing method (Section 4.3.2) was efficient. As might be anticipated, more HPMC area was detected in the tablets containing a higher amount of HPMC. In the formulations containing 7.5 and 12.5 %w/w of HPMC, the HPMC particles look discrete and similar to the shape of their raw materials observed by SEM (Figure 3.2A, Section 3.4.1), demonstrating that there was not much change in the powder mixtures after compaction. When the content of HPMC

was 15 %w/w or higher in the matrices, there were networks and clusters of HPMC. At low content (< 15 %w/w), the HPMC particles are observed to be distant from each other. With increasing the polymer level to 20 %w/w, the HPMC particles seem to be interconnected. A phase transition from individual distinct particles to clusters and network of HPMC was then observed. This could be explained by the findings from previous studies that HPMC particles represented predominantly plastic materials.²⁴⁹⁻²⁵⁰ As a glassy polymer, HPMC is likely to deform plastically during tableting as the stress is applied. The association between the numbers of contact points between the individual particles and the resistance to deformation was previously reported in the literature.²⁵¹ It is probable that increasing the HPMC level leads to a greater number of contact points and less resistance to plastic deformation. Observing the distribution of pharmaceutical materials in the matrices could explain the connection between the formulation variables and their mechanical behaviour.

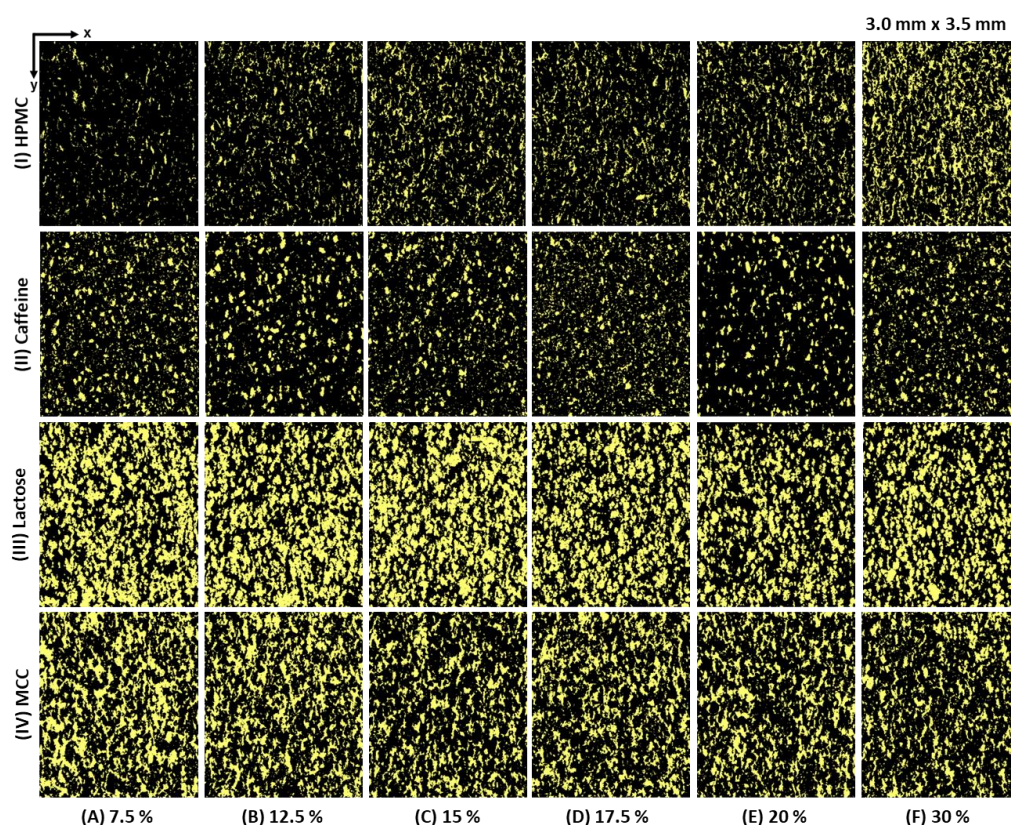


Figure 4.3. Raman maps showing the distribution of (I) HPMC, (II) caffeine, (III) lactose and (IV) MCC in the cross-sectioned tablets containing 7.5 to 30 %w/w HPMC (A to F) with < 125 μm of HPMC particle size fraction.

The regions occupied by caffeine in the Raman maps of the tablet samples (Figure 4.3II) are similar to caffeine clusters which exhibited multiple shapes and sizes as shown in the SEM images of the caffeine raw material (Figure 3.1, Section 3.4.1). The similar morphology of caffeine particles in the Raman map compared to the raw materials confirms that the yellow regions are certainly caffeine. It can be speculated that caffeine might have some resistance to plastic deformation produced during compression. This behaviour of caffeine may be attributed to low compaction pressure used in this study (150 MPa). However, identification of each material by comparing the morphology of raw materials and their morphology in mixtures cannot be done in all cases, especially when the deformation of materials tends to occur. This can be observed in the cases of lactose and MCC. The spray dried lactose raw material occurs as white globular particles (Figure 3.2, Section 3.4.1). After powder mixtures were compressed into tablets, the original lactose particles were hardly detected with Raman imaging (Figure 4.3III). This is in accord with previous studies which indicated that spray dried lactose particles were plastically deforming materials.^{188, 252} Deformation of materials can also be observed in MCC. The SEM micrograph of MCC demonstrated that the long fibrous particles of MCC are similar to the morphology of HPMC raw materials (Figure 3.2). However, MCC became clearly consolidated after tablet compression as shown in the Raman images (Figure 4.3III). This characteristic of MCC in the matrix tablets are also in line with those of previous studies which indicated that MCC mainly exhibited plastic deformation when they consolidate²⁵³⁻²⁵⁵ and MCC have a relatively greater propensity for plastic deformation to the applied load than HPMC.²⁴⁹

The distribution of HPMC particles is the main focus of this study as it may explain the relationship between the formulation variables and the release of caffeine. As mentioned in Section 1.5, the distribution of the polymer has not been investigated yet, especially for the hydrophilic matrix systems. This section has given the general observation of changes in the hydrophilic matrices as a function of the concentration of the polymer in relation to the mechanical properties of each pharmaceutical materials. The analysis with the focus upon the HPMC in relation to caffeine release is shown later in Section 4.4.4.

4.4.2 Component distribution of cross-sectioned tablets using static ToF-SIMS: effect of polymer concentration

The nature of CRM and static ToF-SIMS acquisitions is non-destructive, as a consequence, these techniques can be used to analyse a coincident area of the same sample. CRM is comparatively more popular than ToF-SIMS. The advantages of ToF-SIMS in chemical imaging over CRM including higher spatial resolutions, surface sensitivity and acquisition time, have been discussed in 2.2.9. Using the diagnostic secondary ions identified for caffeine and tablet excipients shown in Section 3.4.4.1, the cross-sectioned tablet samples were analysed. Samples containing 7.5 and 30 %w/w HPMC with less than 125 μm of the HPMC particle size fraction are illustrated in Figure 4.4. It was found that caffeine, HPMC and lactose were homogeneously mixed. HPMC characteristics are significantly different between each formulation. The long fibrous particles of HPMC can be observed in Figure 4.4A, as displayed as examples with white arrows. HPMC particles were connected to each other to form percolating networks (as highlighted by white arrows in Figure 4.4B) with regard to the higher content in the 30 %w/w. The black area may denote unidentifiable MCC or any void space in the tablets. Compared to Raman microscopy, MCC particles were not diagnosed using ToF-SIMS. As mentioned in Section 3.4.4.1, their peaks were overlapped with other materials. This indicates the insufficient mass resolution of ToF-SIMS. It could be suggested that other techniques may compensate for a lack of sufficient mass resolution. Such technique includes 3D OrbiSIMS, which is the combination between the high spatial resolution of SIMS and the high mass resolution of an OrbitrapTM. It would allow visualisation of materials in the tablet with higher mass-resolving power than ToF-SIMS. 3D OrbiSIMS could possibly resolve MCC. As the study focuses on the distribution and characteristics of HPMC particles, ToF-SIMS then provide comparable results to Raman regarding HPMC distribution.

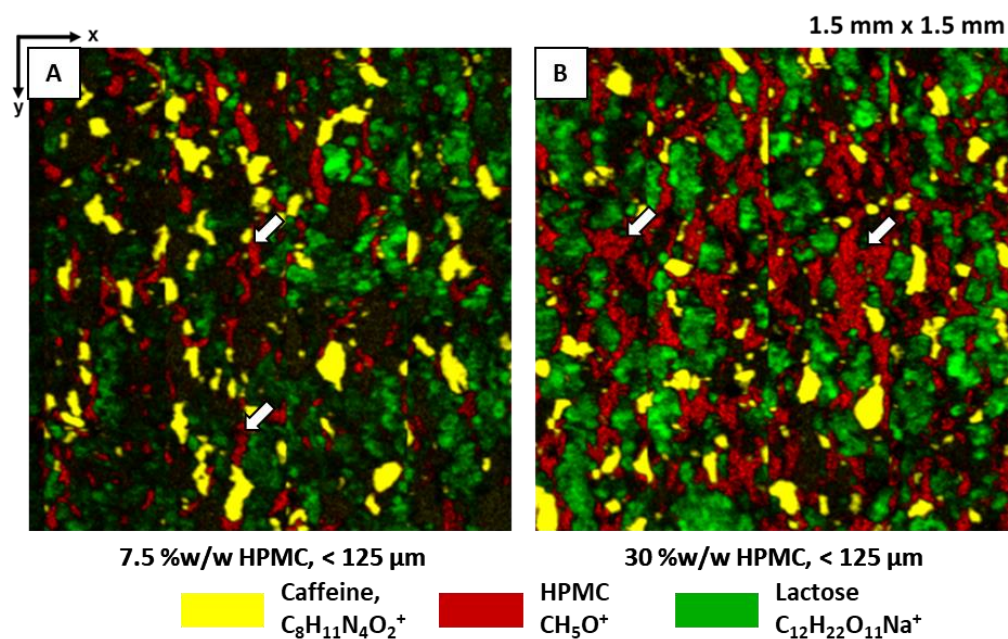


Figure 4.4. Representative ToF-SIMS images showing the comparison between the cross-sectioned tablets containing 7.5 and 30 %w/w HPMC with <125 μm of HPMC particle size fraction representing caffeine (yellow), HPMC (red) and lactose (green). White arrows highlight different characteristics of HPMC. Data taken from the tablets with the diameter of 8 mm and thickness of 4 mm.

Each individual component in the tablets was chemically imaged using ToF-SIMS, as depicted in Figure 4.5. The area of HPMC particles was significantly increased as the polymer concentration increased, especially in the 30 %w/w. The connected networks were found in the formulations with 12.5 %w/w HPMC or higher. Caffeine particles were clearly identified as clusters and powders, similar to the SEM data (Figure 3.1). This similarity of caffeine assists in the identification of caffeine in the tablets. The deformation of lactose was generally observed in all formulas and similar to the Raman data (Section 4.4.1).

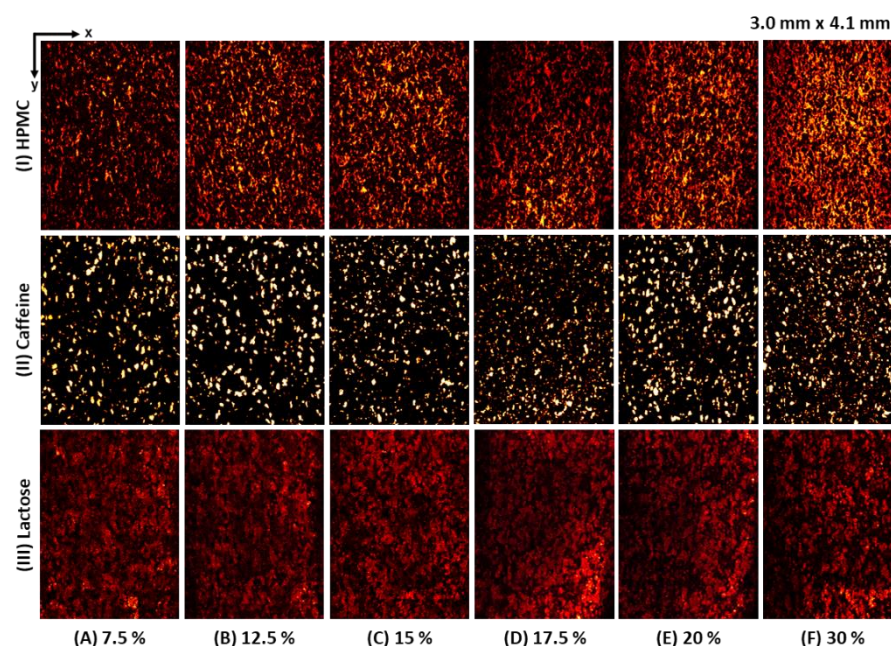


Figure 4.5. ToF-SIMS images showing the distribution of (I) HPMC, (II) caffeine and (III) lactose in the cross-sectioned tablets containing 7.5 to 30 %w/w HPMC (A to F) with $<125\ \mu\text{m}$ of HPMC particle size fraction.

Raman and ToF-SIMS data are compared in Figure 4.6. It is found that in the same analysed areas the results from ToF-SIMS are consistent with data obtained from CRM, especially the location, distribution pattern and size of each particle. In Figure 4.6A and B, the distribution of all components is in a similar position as indicated for example by Circle 1, 2 and 3 for the distribution of caffeine, HPMC and lactose respectively. The chemical images of HPMC gained by Raman (Figure 4.6C) and ToF-SIMS (Figure 4.6D) demonstrate the similar positions of HPMC particles. The small differences in particle characteristics between Raman and ToF-SIMS images can be noticed and they are highlighted with red circles in Figure 4.6C and D. However, the analytical depths between two techniques are not the same. As commonly known, the primary ion beam of ToF-SIMS focuses on the first few layers of the sample's surface which are approximately 1 nm of average analytical depth. Raman laser can penetrate the sample's surface deeper down to 1 μm . Their respective plot profiles, which are the line graph of the averaged pixel intensity plotted against the horizontal distance, were obtained using ImageJ as shown in Figure 4.6E and F. They demonstrate that the HPMC particles from both techniques were distributed in

the same patterns and the HPMC particles acquired were in a similar position (grey circles). The findings indicate that ToF-SIMS and CRM can validate the data from each other. ToF-SIMS then could be an analytical technique of choice for visualising the chemical distribution of tablet components.

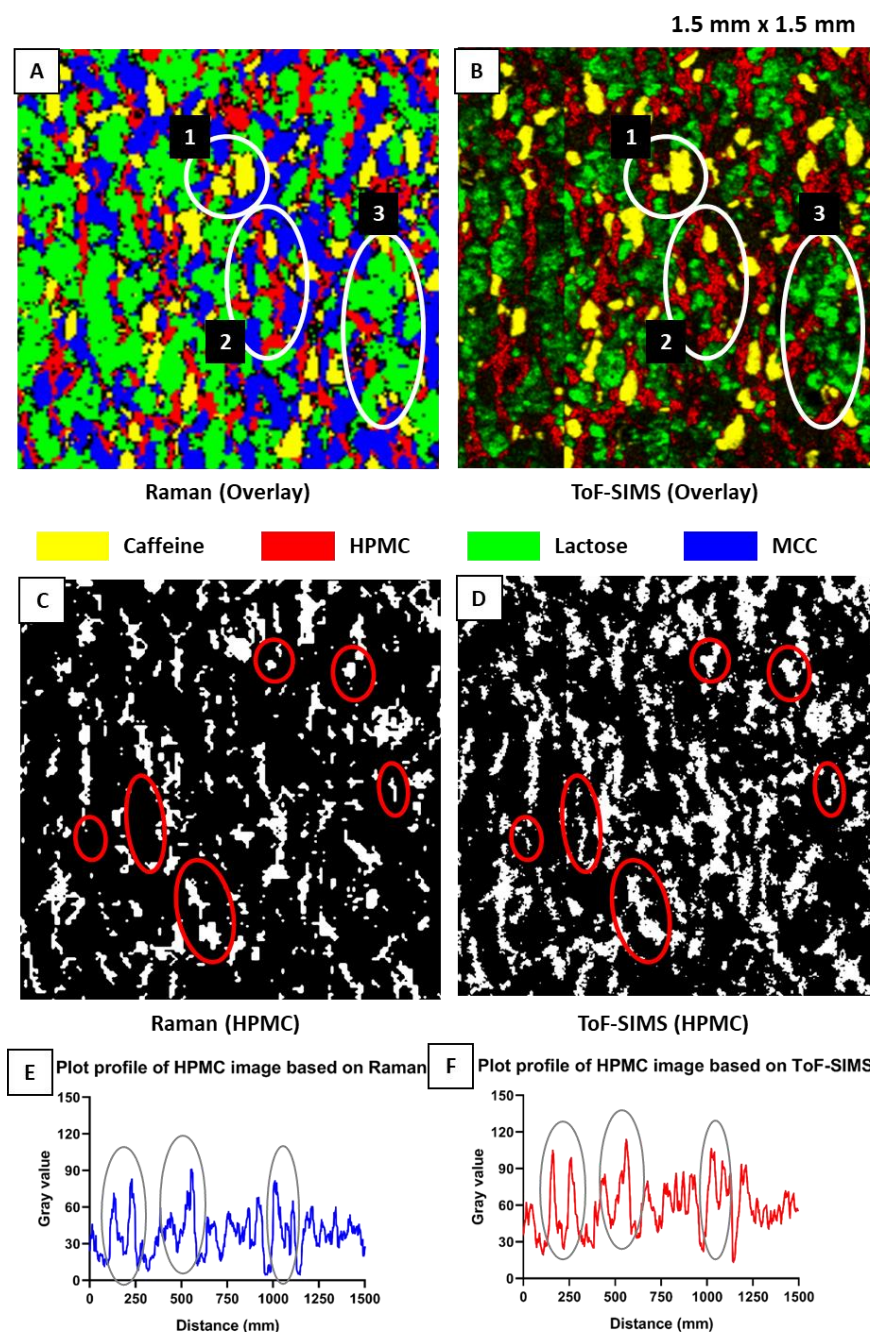


Figure 4.6. Comparison between Raman and ToF-SIMS images: (A) Raman overlay, (B) ToF-SIMS overlay, (C) Raman image of HPMC, (D) ToF-SIMS image of HPMC, (E) Plot profile of HPMC particles based on Raman data and (F) Plot profile of HPMC particles based on ToF-SIMS. White circles highlight similar locations of caffeine (1), HPMC (2) and lactose (3).

4.4.3 Component distribution of cross-sectioned tablets using static ToF-SIMS: effect of polymer particle size

The particle size of HPMC polymers is a formulation factor that influences *in vitro* release performance of an active substance from matrix tablets even if its effect becomes less sensitive with increasing HPMC content. To study the effect of HPMC particle size on the tablet microstructure, tablets containing 15 %w/w HPMC with varied HPMC particle size fractions from smaller than 45 to larger than 125 μm were also microtomed and chemically mapped using ToF-SIMS.

As shown in Figure 4.7, it is apparent that the detected size of HPMC particles in the matrix was different as expected. The tablet with HPMC particle size fraction < 45 μm (Figure 4.7A) shows a large number of fine HPMC particles in accordance with its known size. At reduced polymer particle size, the HPMC particles appear mutually attached demonstrating the networks of HPMC particles in the matrix. White arrows in Figure 4.7A highlight the area where HPMC particles were connected. One of HPMC detected particles in the matrix was found to be about 100 μm in length which is much larger than the original size of HPMC raw materials. This demonstrates that HPMC connected particles were formed. This is probably due to the fact that the surface area of HPMC is increased with reducing the particle size, thus increasing the chance of polymer attachment and swelling. After exposure to water, these HPMC clusters would swell and later provide more of a physical barrier to drug release, compared to the tablet with over the 125 μm of HPMC particle size fraction. The particle size of the HPMC may impact the localisation of caffeine during dissolution. While the reduced HPMC fraction shows the increased number of HPMC networks, the tablet with the particle size fraction of > 125 μm shows more space in-between particles (Figure 4.7B). The detected particle size of about 300 μm is even found in the > 125 μm formula (Figure 4.7B). As discussed in Section 4.4.2, the clusters of HPMC networks are progressively found when the concentration of HPMC increases, especially in the 30%w/w HPMC tablets with the particle size fraction of < 125 μm . However, ToF-SIMS chemical imaging proved that the network of HPMC can even be observed in the tablets containing 15% of

HPMC with reduced size fractions i.e. $< 45 \mu\text{m}$. Therefore, the particle size of HPMC is also important to the release behaviour of drug from the hydrophilic matrices.

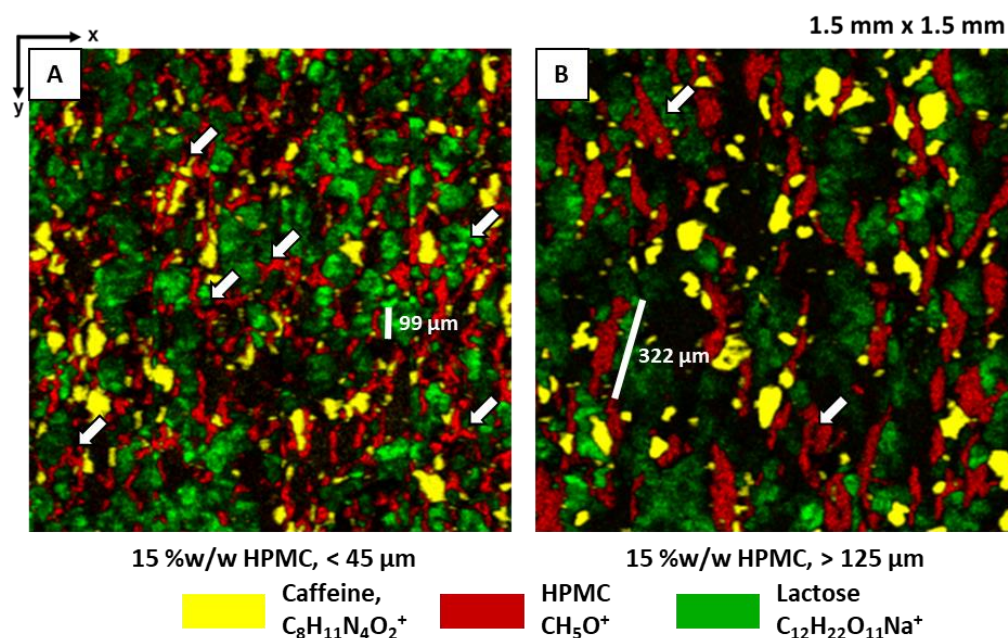


Figure 4.7. Representative ToF-SIMS images showing the comparison between the cross-sectioned tablets containing 15 %w/w HPMC with < 45 and $> 125 \mu\text{m}$ of HPMC particle size fraction representing caffeine (yellow), HPMC (red) and lactose (green). Data taken from the tablets with the diameter of 8 mm and thickness of 4 mm.

The smaller the particle size of HPMC was incorporated, the more the networks of HPMC were observed. The HPMC distribution images (Figure 4.8I) shows that increasing the HPMC particle size led to the individually separate distribution of HPMC particles, as illustrated in Figure 4.7 and Figure 4.8.

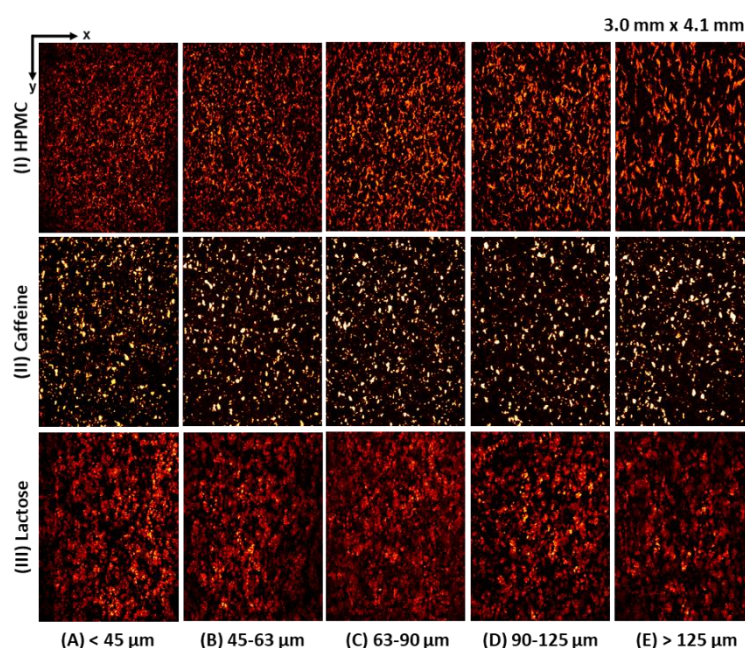


Figure 4.8. ToF-SIMS images showing the distribution of (I) HPMC, (II) caffeine and (III) lactose in the cross-sectioned tablets containing 15 %w/w HPMC with < 45 to > 125 (A to F) μm of HPMC particle size fraction.

The angle between the Feret's diameter and a line parallel to the x-axis of the image was calculated and reported as Feret angle or particle angle. The histograms of particle angle values (Figure 4.9) were produced and fitted with the Gaussian distribution (i.e. normal distribution). It can be seen that in the formulations with smaller particle size, the HPMC particles were aligned randomly (Figure 4.9A). Interestingly, when the large particle size fractions were used, the orientation of the HPMC particles are found to be parallel to the y-axis, especially the > 125 μm formulation (Figure 4.8EI and Figure 4.9E). This orientation is in agreement with the compression direction of tablets, i.e. normal to the direction of the compressive force. This typical arrangement of HPMC particles may affect the development of the gel after hydration.

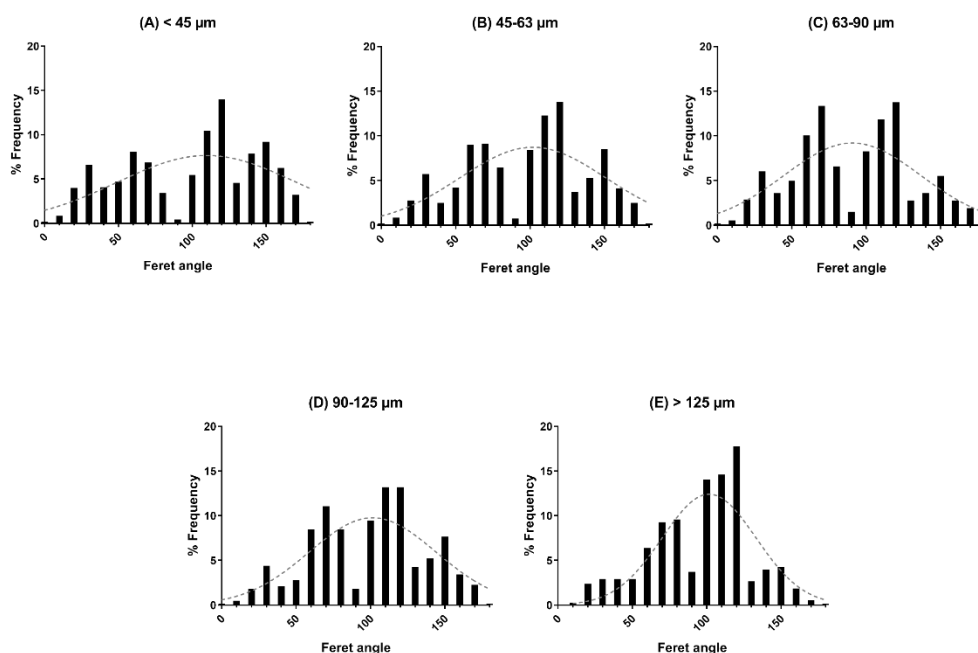


Figure 4.9. Histograms of Feret angle of HPMC particles with fitted Gaussian distributions (grey dashed lines) in the cross-sectioned tablets containing 15 %w/w HPMC with < 45 to > 125 (A to F) μm of HPMC particle size fraction.

4.4.4 Quantitative analysis of particle characteristics and distributions

As discussed in Section 4.4.2 and 4.4.3, the particle characteristics and distribution of HPMC were changed as a function of HPMC concentrations and particle size fractions. A series of CH_5O^+ secondary ion images (3 replicates) representing HPMC (from both HPMC content and particle size experiments) were converted to binary images using ImageJ software (Section 4.3.7) for quantitative analysis. The particle count, area and perimeter, distribution and orientation of HPMC particles were correlated with the formulation variables.

4.4.4.1 HPMC particle characteristics

As shown in Figure 4.10, the characteristics of HPMC particles include particle counts, particle area and particle perimeter. Increasing the HPMC concentration from 7.5 to 30 %w/w had a negligible impact on particle count as the number of HPMC particles increased by 300 particles from 1000 to 1300 particles per 2.2

mm $\times$ 4.1 mm (Figure 4.10A). Taking the chemical images in Figure 4.5I into account, it can be observed that the HPMC became connected to each other, thus affecting the detection of particles. The chance in detecting the particles is higher when the content of HPMC is increased but balanced against the greater number of connected clusters. However, when the concentration of HPMC was fixed at 15 %w/w, the detected particle count significantly reduced from 1500 to 500 particles (Figure 4.8). The smaller the size of the HPMC was, the greater the chance in detecting the particles were found. The content of 15 %w/w did not produce a great number of percolated clusters (Figure 4.8I). Therefore, the percolated clusters do not highly impact the particles detected. Particle size then more considerably affects the particle count than the concentration of HPMC. In the case of the size of HPMC, the parameters used to reflect the particle sizes are particle area and perimeter. They were selected due to the fact that these parameters are not affected by the shape of the particles, unlike the particle length. As expected, there were a positive relationship between detected particle size in the matrices and the content of HPMC used in the formulation and the particle size fractions of HPMC raw materials (Figure 4.10C – F). The particle size had a more considerable impact on the particle area (Figure 4.10D) and perimeter (Figure 4.10F) of detected HPMC than HPMC concentration. In the concentration series, the fraction of the small HPMC may be relatively higher than the large HPMC. In other words, the particle size distribution may be right skewed (positively skewed) between 0 to 125 μ m. Hence, right skewness and wide deviation of particle size may impact the average values to be lower than it should be in the normal distribution.

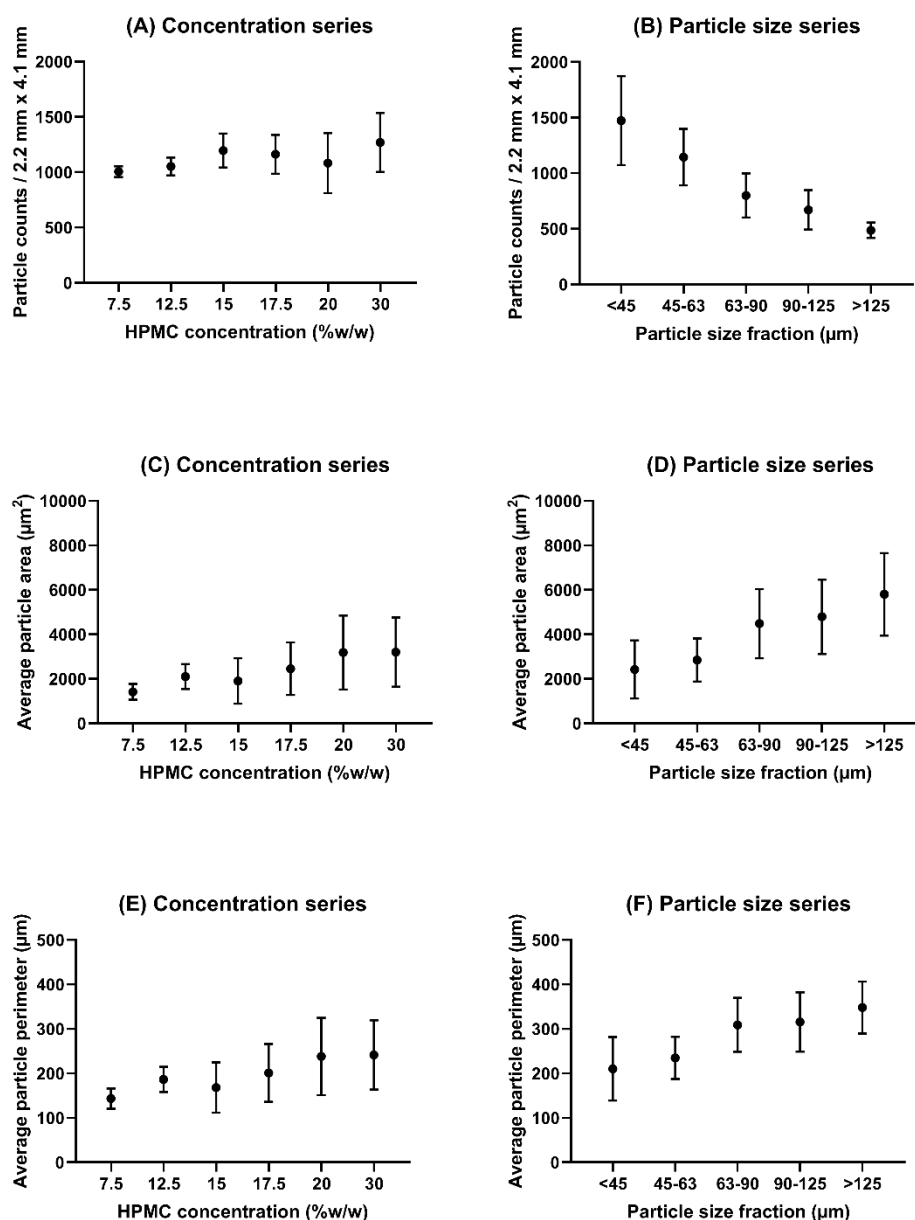


Figure 4.10. Particle characteristics of HPMC particles (mean $\pm$ SD) as a function of HPMC concentration and particle size: (A) particle count from concentration series, (B) particle count from particle size series, (C) Average particle area from concentration series, (D) Average particle area from particle size series, (E) Average particle perimeter from concentration series and (F) Average particle perimeter from particle size series.

4.4.4.2 Distribution of HPMC

In order to quantify how much the connectivity of the HPMC particles occurred as a function of formulation variables including polymer level and particle size,

the ToF-SIMS chemical images of HPMC were converted to skeletonised binary images (Figure 4.11A and B). The steps to image processing and extracting important particle parameters can be found in Section 4.3.7. The skeletonised features of the HPMC are highly consistent with the amount of HPMC in the formulation. It is clearly shown in skeletonised images of the 30 %w/w HPMC tablet (Figure 4.11B) that the HPMC particles became percolated more densely whereas they were significantly less connected in the 7.5 %w/w HPMC tablet (Figure 4.11A).

The comparison between the intersection maps of 7.5 and 30 %w/w HPMC tablets can be seen as examples in Figure 4.11C and D. Locations where the HPMC particles were interconnected are indicated by red dots. The HPMC particles are generally long, fibrous (Section 3.4.1). Consequently, it can be assumed that the branched skeletons of HPMC particles can represent HPMC connectivity. The comparative degree of HPMC continuous networks among all formulations can be estimated by how many intersections detected in each skeletonised image. The relationship between HPMC concentration and the number of intersections was observed as shown in Figure 4.11E. A significant increase in the number of intersections from less than 300 to almost 1500 was found in matrices containing an increasing amount of HPMC.

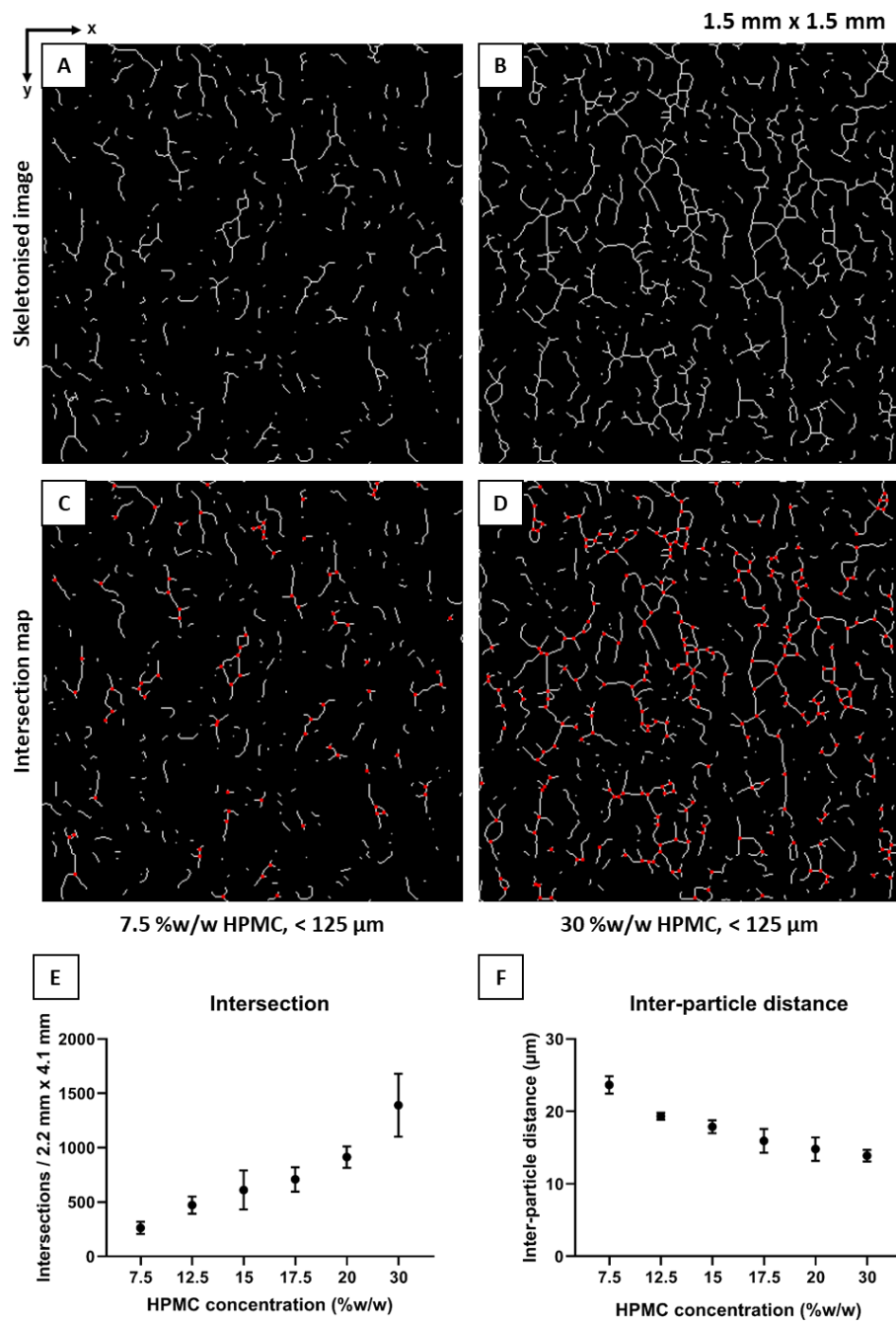


Figure 4.11. Skeletonised and intersection maps showing the distribution and connectivity of HPMC particles: (A) skeletonised HPMC image of 7.5 %w/w HPMC tablet, (B) skeletonised HPMC image of 30 %w/w HPMC tablet, (C) intersection map of 7.5 %w/w HPMC tablet, (D) intersection map of 30 %w/w HPMC tablet, (E) intersection count of concentration series and (F) inter-particle distance of concentration series. Red dots highlight points where HPMC particles are interconnected.

Another parameter acquired from the binary image that could reflect the distribution of HPMC particles is the inter-particle distance. It is the averaged shortest distance between each pair of HPMC particles calculated based on the HPMC binary image using the ‘Graph’ plugin as previously mentioned in Section 4.3.7. As anticipated, it can be seen in Figure 4.11F that using the higher amount of HPMC in the formulation led to the reduced inter-particle distance, demonstrating that the particles became closer to form clusters. The reduction of the inter-particle distance when the content of HPMC was higher may show that there was the percolation network formation within the matrices.

Figure 4.12A - D show the interconnectivity of the matrices containing 15 %w/w HPMC with different particle size fractions. The skeletons of the tablet with < 45 μm (Figure 4.12A) were more crowded than those with larger particles (Figure 4.12B). With 15 %w/w of HPMC and < 45 μm of particle size fraction, the skeletons were almost fully distributed. The intersection detection of tablets was also done using ImageJ as mentioned in Section 4.3.7. It was found that when changing the particle fraction from < 45 to > 125 μm the number of intersections decreased from roughly 800 to 300 (Figure 4.12E). The graph in Figure 4.12F illustrates the relationship between the particle size of HPMC used and its inter-particle distance. The inter-particle distance became longer if the longer fibrous particles of HPMC were incorporated in the formulation. This is in agreement with the percolation theory that components with smaller particle size show higher efficiency to interconnect. More attachment in the systems with reduced particle size could be explained by the reason that the surface area of the particle is increased. This parameter could represent the connectivity of the HPMC particles in the formulation.

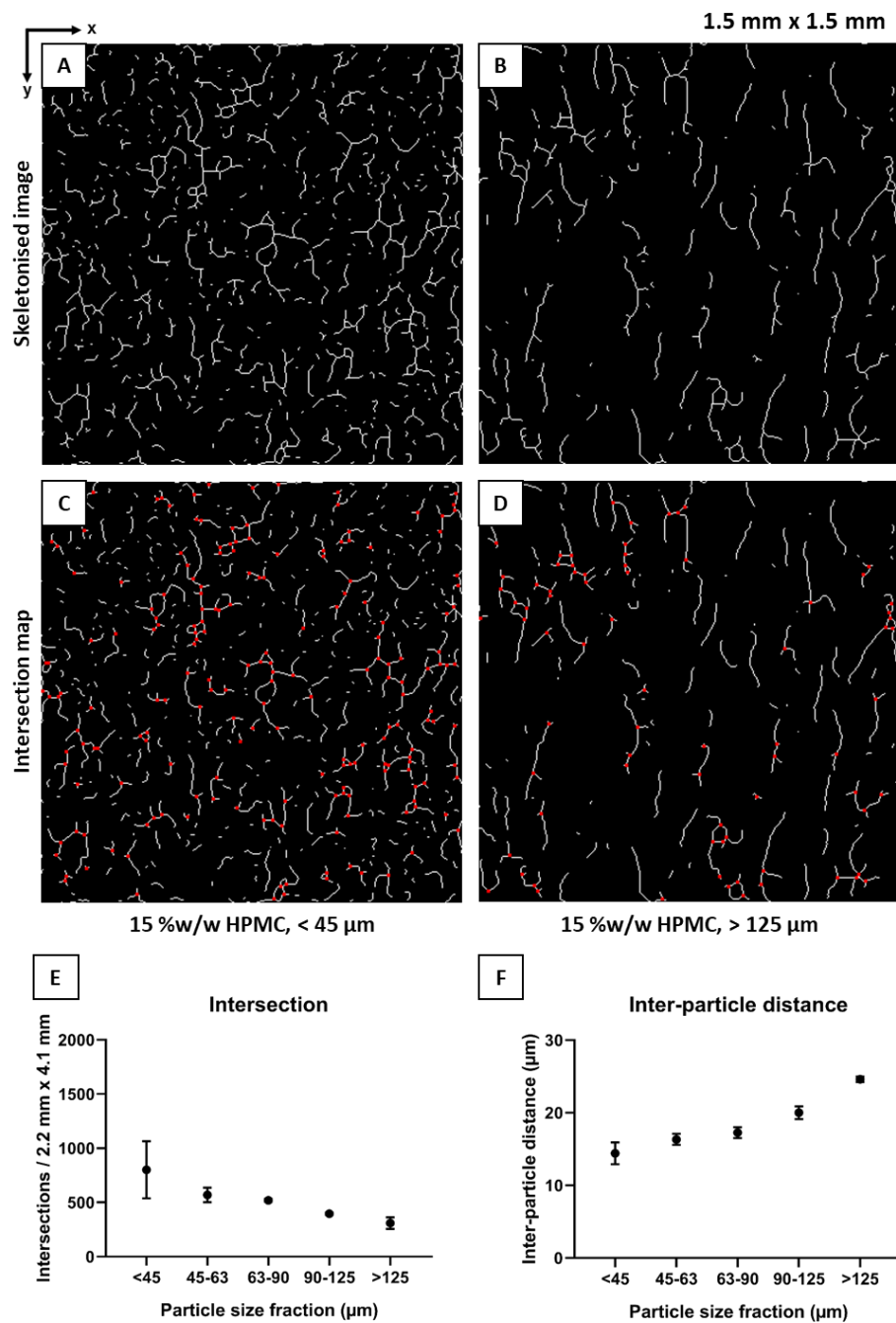


Figure 4.12. Skeletonised and intersection maps showing the distribution and connectivity of HPMC particles: (A) skeletonised HPMC image of < 45 μm HPMC tablet, (B) skeletonised HPMC image of > 125 μm HPMC tablet, (C) intersection map of < 45 μm HPMC tablet, (D) intersection map of > 125 μm HPMC tablet, (E) intersection count of particle size series and (F) inter-particle distance of concentration series. Red dots highlight points where HPMC particles are interconnected.

To conclude, the distribution of HPMC was influenced by formulation factors which is considered as critically important for the dissolution performance. It could be assumed that in the formulations with less connected HPMC particles would develop less gel barriers and a greater possibility of water could more quickly hydrate the matrices. This might contribute to an initial burst release of caffeine, incomplete gel layer development and accordingly a rapid dissolution rate. The more complex skeletonised networks observed in the 30 %w/w HPMC tablet than the 7.5 %w/w could indicate that the formation of a gel layer is possibly generated leading to entrapment of caffeine particles in the matrices. Likewise, this effect also would be expected from the level of network complexity found in the reduced particle fraction. It is important to find a relationship between the networks of HPMC and the overall dissolution of caffeine.

4.4.5 Predicting the dissolution using HPMC parameter-based regression models

4.4.5.1 Correlating HPMC parameters with drug release performance

An inverse relationship has been demonstrated to exist between the concentration of HPMC and the dissolution of the active agent as described in Section 1.2.5.2. Additionally, in terms of particle size, a more delayed dissolution rate was found in a formulation with fine HPMC particles. Section 4.4.4 shows that the characteristics of HPMC particles, their distribution and connectivity were influenced by the formulation factors. In order to investigate this phenomenon the chemical imaging data acquired using ToF-SIMS analysis was used to interrogate a series of HPMC particle parameters seeking to identify correlations with caffeine release data. The single point dissolution at 30 minutes was chosen to represent the early dissolution of caffeine from the HPMC matrices. This time point was also not too quick to demonstrate large deviation in dissolution rates. In order to do this a series of CH_5O^+ secondary ion images (3 replicates) representing HPMC (from both HPMC content and particle size experiments) were converted to binary images using ImageJ software (Section 4.3.7). Using these image data-sets, a range of HPMC particle characteristics

were analysed and exported including the shortest distance between HPMC particles, particle count, particle perimeter, particle area and length.

Figure 4.13 illustrates the graph between HPMC parameters calculated based upon CH_5O^+ secondary ion images including the inter-particle distance (Figure 4.13A), intersection (Figure 4.13B), count (Figure 4.13C), sum of particle perimeter (Figure 4.13D), particle perimeter (Figure 4.13E), sum of particle area (Figure 4.13F), particle area (Figure 4.13G), Feret diameter (Figure 4.13H) and minimum Feret diameter (Figure 4.13I). The results for which were illustrated as a function of the dissolution of caffeine at 30 minutes. Pearson's correlation coefficients (r) between each parameter and the dissolution were calculated to measure the strength and direction of a linear relationship between each pair of variables which are presented in Table 4.1. The r values are an effective way to measure the linear correlative degree between two variables. r values of zero indicate that no linear relationship exists between the two variables, whereas a coefficient of -1 or $+1$ indicates a perfectly linear relationship. The stronger the correlation, the closer the correlation coefficient is to ± 1 . If the coefficient is a positive number, there is a direct relationship between the two variables. Contrarily, if the coefficient is in negative, the variables are inversely related. After, calculating the r value, the HPMC parameters can be categorised into three groups as follows, concentration dependent variables, particle size dependent variables and highly correlated variables.

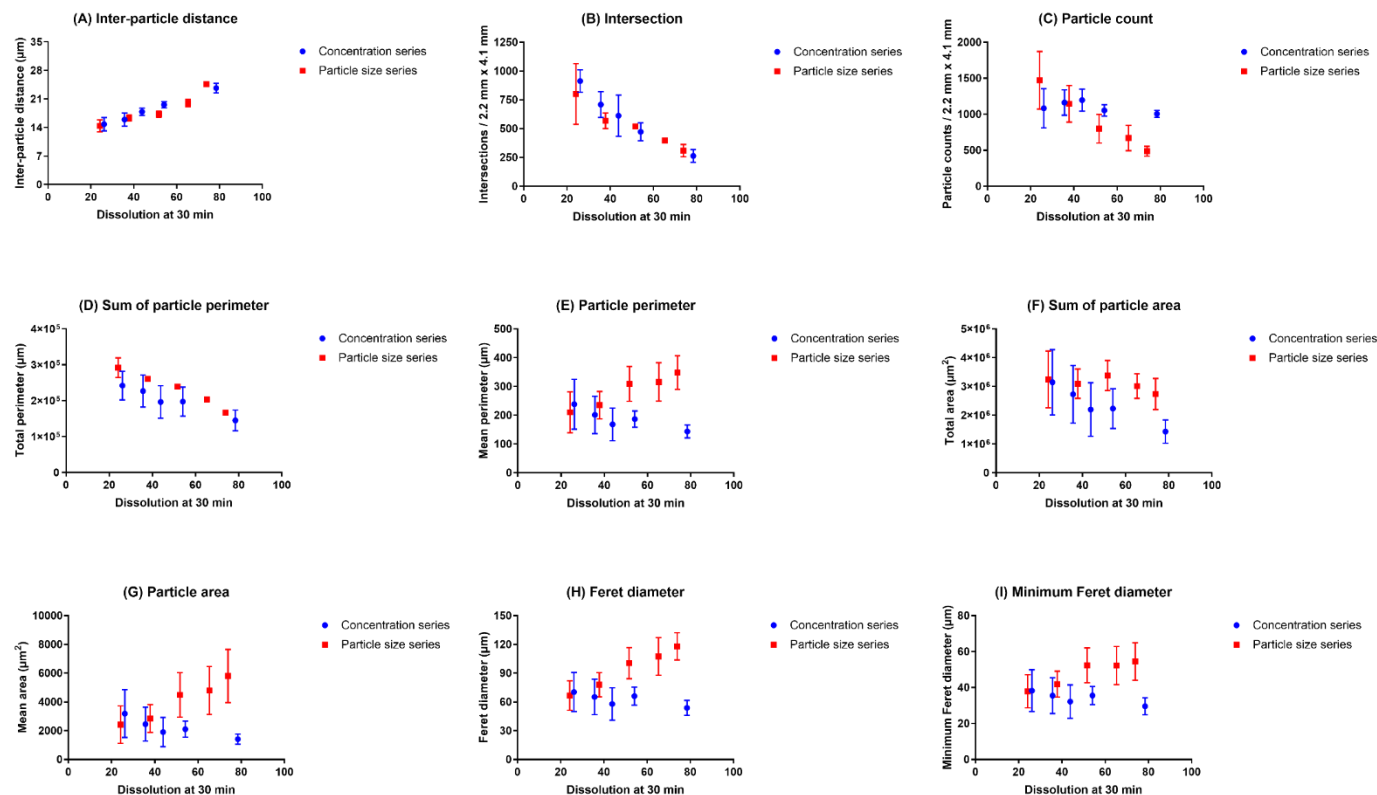


Figure 4.13. HPMC parameters based on CH_5^+ ion images including (A) inter-particle distance, (B) intersection, (C) particle count, (D) sum of particle perimeter, (E) particle perimeter, (F) sum of particle area, (G) Particle area, (H) Feret diameter and (I) minimum Feret diameter acquired from the HPMC concentration and particle size series.

Table 4.1. Pearson's correlation coefficients calculated between the image parameters from concentration and particle size series and the dissolution at 30 min. The *p* values are in parentheses.

Image parameter	Concentration series	Particle size series	Group
(A) Inter-particle distance	0.9981 (<0.0001)****	0.9389 (0.0180)*	Both
(B) Intersection	-0.9815 (0.0030)**	-0.9773 (0.0041)**	Both
(C) Particle count	-0.6262 (0.2584)	-0.9891 (0.0014)**	Particle size
(D) Sum of particle perimeter	-0.9803 (0.0033)**	-0.9869 (0.0018)**	Both
(E) Particle perimeter	-0.8899 (0.0431)*	0.9716 (0.0057)**	Both
(F) Sum of area	-0.9699 (0.0062)**	-0.6697 (0.2162)	Concentration
(G) Particle area	-0.9041 (0.0351)*	0.9766 (0.0043)**	Both
(H) Feret diameter / Particle length	-0.7935 (0.1091)	0.9890 (0.0014)**	Particle size
(I) Minimum Feret diameter / Particle width	-0.8457 (0.0710)	0.9473 (0.0144)*	Particle size

* refers to p value ≤ 0.05 , ** refers to p value ≤ 0.01 , *** refers to p value ≤ 0.001 and **** refers to p value ≤ 0.0001 .

As shown in Table 4.1A, *R* for the inter-particle distance was positive and higher than 0.93 ($p \leq 0.05$) for both concentration and particle size series indicating that there was a significantly strong positive linear correlation between the inter-particle distance and drug release (Figure 4.13A). The inter-particle distance is the average of the closest distance between each pair of HPMC neighbouring particles. This parameter could directly refer to the possibility of HPMC particles forming a continuous complete gel layer, thereby correlating the dissolution. It

will be less likely to form a complete gel if the inter-particle distance is great. This is in line with a percolation threshold at which the formation of a drug-retarding network is developed (Section 1.2.6).

The connectivity of HPMC networks is thought to exert an important control on drug release in pharmaceutical tablets. The number of intersections is another parameter which involves the distribution of HPMC by calculating how many interconnected HPMC particles there are in the tablet's cross-sections. It can be seen in Figure 4.13B and Table 4.1B that the number of intersections shows highly negatively correlated relationships with the dissolution of caffeine from the matrix tablets at 30 minutes ($r < -0.98$, $p \leq 0.01$). This clearly demonstrates that the matrices containing a higher number of interconnected HPMC networks exhibited a more delayed drug release.

As shown in Figure 4.13D and Table 4.1D, the relationship between the dissolution and sum of the perimeter is strongly negatively correlated, as evidenced by the r values of less than -0.98 ($p \leq 0.01$). The observed correlation between the sum of perimeter and drug release might be explained as follows; particle perimeter is particle circumference which is a 2D parameter which very likely represents the surface area of HPMC in 3D. Sum of particle perimeter then could refer to the total surface area which possibly indicates the capacity of dried HPMC particles to become hydrated and form effective gels.

The calculated r values between the particle perimeter/area and dissolution rate were also close to -1 or 1 (Table 4.1E, G, Figure 4.13E and G). These variables corresponded to the particle size fractions in a direct manner. However, they were inversely related to the tablets with varied HPMC concentrations. It may be explained by the fact that when the content of HPMC was increased, the particles became interconnected to form the cluster/networks. Correspondingly, the number of observed clusters/networks observed were greater, while the number of small particles was reduced. Therefore, increasing the content of HPMC results in the reduction of the average circumference and area of particles. The average perimeter and area can be varied depending on the particle size fractions used.

A concentration dependent variable was the sum of the particle area as can be seen in Table 4.1F and Figure 4.13F. In the concentration series, there is a linear relationship between the sum of particle area and caffeine release with a high r value of -0.97 (p value ≤ 0.01). However, the sum of the particle area was not significantly related to dissolution observations from the particle size series ($R = -0.7$, p value > 0.05). The r and p values suggest that the sum of the particle area does not universally relate to drug release. Sum of the area is calculated by adding up the overall area that HPMC particles occupy. As anticipated, the higher amount of HPMC in the formulation, the higher chance of HPMC being observed in the cross-sectioned plane. However, the observations may depend on the projection direction of the cross-section and the alignment of HPMC particles. If the HPMC particles were not uniformly distributed in the tablets or the particles did not align in one direction, the chance where the r value is high would be little.

As shown in Table 4.1C, the particle count (Figure 4.13C), exhibited a non-significant correlation ($r = -0.6$, $p > 0.05$) with dissolution performance from the concentration series. In contrast to the concentration series, this variable was significantly dependant on data from particle size ($r < -0.95$, $p \leq 0.01$). It may be explained by the fact that using higher polymer content may exhibit polymer aggregates or networks rather than individual particles in dry matrices, as shown in Figure 4.4. ImageJ then was unable to detect fine particles, resulting in reduced particles detected. This also results in the increased mean perimeter and mean area of HPMC particles even if the particle size fractions were constant among all batches in the concentration series.

Feret diameter and minimum Feret diameter are the maximum and minimum size of the objects using a caliper. In other words, Feret diameter represents particle length and minimum Feret diameter represents particle width. Feret diameter (Figure 4.13H) and minimum Feret diameter (Figure 4.13I) were also only related to the data from the particle size as shown in Table 4.1H and I that p values are above 0.05. This demonstrates that they are not the critical parameters which could describe the dissolution.

Parameters calculated by ToF-SIMS chemical imaging including, particle count, the sum of particle area, particle length and width were not correlated with the dissolution from both series of formulation factors. This demonstrates that these parameters are non-indicative of explaining the dissolution behaviour of caffeine at 30 minutes. Thus, the inter-particle distance, intersection count, sum of the perimeter, mean perimeter and mean area could be possibly critical HPMC parameters for understanding a link between the formulation factors and drug dissolution.

4.4.5.2 Building and assessing the performance of simple/multiple linear regression models

To determine the strength of the relationship between one dependent variable and a series of independent variables, regression analysis was done. There are five variables potentially useful for mathematical modelling, as discussed in Section 4.4.4, including inter-particle distance, the number of intersections, sum of the perimeter, particle perimeter and area. According to the ‘rule of thumb’ for interpreting the size of a correlation coefficient suggested by Hinkle *et al.*²³², a correlation coefficient of 0.90 to 1.00 or -1.00 to -0.90 is considered as a very high positive or negative correlation. In this section, only the associations which have the correlation coefficient in a range of 0.90 to 1.00 or -1.00 to -0.90 were taken for mathematical models. Particle perimeter then was excluded from the regression analysis as R is more than -0.90 in the concentration series (Table 4.1E).

Linear regression models based on one (simple regression) or more image parameters (multiple regression) were developed to predict the dissolution of caffeine at 30 minutes (y_{30}), where x_1 is the inter-particle distance of HPMC particles (μm), x_2 is the number of intersections, x_3 is sum of the perimeter (μm) and x_4 is the mean area of HPMC particles (μm^2). Regarding four independent variables, there are 15 possible linear regression equations (Table 4.2). Statistical indicators were required to compare regression models and determine the predictive power and accuracy of these models.

Table 4.2. Simple and multiple linear regression models for predicting caffeine release at 30 minutes (y_{30}) with statistical parameters.

No.	Simple/multiple linear regression model	R^2	$\bar{R}^2$	MAE
1	$y_{30} = 5.2123x_1 - 47.0282$	0.9272	0.9181	3.7935
2	$y_{30} = -0.08771x_2 + 97.9210$	0.9331	0.9248	3.4325
3	$y_{30} = -0.0003767x_3 + 130.8042$	0.7604	0.7304	7.7652
4	$y_{30} = 0.004173x_4 + 36.0251$	0.0977	-0.0151	13.7758
5	$y_{30} = 2.6182x_1 - 0.04736x_2 + 27.1719$	0.9696	0.9609	2.7983
6	$y_{30} = 5.0924x_1 - 0.00001062x_3 - 42.5124$	0.9273	0.9065	3.8008
7	$y_{30} = 5.1548x_1 + 0.0004894x_4 - 47.5048$	0.9284	0.9080	3.7337
8	$y_{30} = -0.06619x_2 - 0.0001315x_3 + 114.4582$	0.9696	0.9609	2.8475
9	$y_{30} = -0.08626x_2 + 0.0007989x_4 + 94.6073$	0.9364	0.9183	3.2554
10	$y_{30} = -0.0003709x_3 + 0.0036x_4 + 118.2201$	0.8333	0.7857	6.6384
11	$y_{30} = 1.4955x_1 - 0.05234x_2 - 0.00007530x_3 + 66.9802$	0.9748	0.9622	2.4739
12	$y_{30} = 2.5603x_1 - 0.04736x_2 + 0.0004910x_4 + 26.7008$	0.9708	0.9562	2.7303
13	$y_{30} = 4.6893x_1 - 0.00003838x_3 + 0.007638x_4 + 31.4559$	0.9295	0.8943	3.8257
14	$y_{30} = -0.05990x_2 - 0.0001522x_3 + 0.001599x_4 + 110.4379$	0.9820	0.9730	2.3082
15	$y_{30} = 0.4784x_1 - 0.05611x_2 - 0.0001322x_3 + 0.001436x_4 + 95.6611$	0.9824	0.9684	0.9037

y_{30} is caffeine release at 30 min (%), x_1 is inter-particle distance (μm), x_2 is intersection / $2.2 \text{ mm} \times 4.1 \text{ mm}$, x_3 is the sum of the perimeter (μm) and x_4 is particle area (μm^2).

Stepwise regression was used as an approach to the selection of significant HPMC parameters in the model as it always re-evaluates each variable in the model. The criteria for the linear regression models are shown in Section 2.3.4

The first step of Stepwise regression is to identify the most significant variable to add to the model. Considering the model with a single parameter (Model 1 - 4), it is apparent from p values in Table 4.2 that the regression model based on the number of intersections (Model 2) has the highest R^2 of 0.9331, demonstrating that 93.31 % of the variation can be explained by the model with the number of intersections. Apart from that, it can be seen from Table 4.3 that this model is significant (p values < 0.0001) with highest F value. The number of intersections is considered as a significant independent variable.

Table 4.3. F and t from ANOVA and student t test respectively with p values in parentheses.

	F	t of x_1	t of x_2	t of x_3	t of x_4
1	101.87 (<0.0001)****	10.09 (<0.0001)****	-	-	-
2	111.61 (<0.0001)****	-	-10.56 (<0.0001)****	-	-
3	25.38 (0.0010)***	-	-	-5.04 (0.0010)***	-
4	0.87 (0.3792)	-	-	-	0.93 (0.3792)
5	111.47 (<0.0001)****	2.89 (0.0232)*	-3.12 (0.0168)*	-	-
6	44.64 (0.0001)****	4.01 (0.0051)**	-	-0.10 (0.9195)	-
7	45.40 (<0.0001)****	9.01 (<0.0001)****	-	-	0.35 (0.7388)
8	111.59 (<0.0001)****	-	-6.94 (0.0002)***	-2.90 (0.0231)*	-
9	51.57 (<0.0001)****	-	-9.61 (<0.0001)****	-	0.61 (0.5640)
10	17.50 (0.0019)**	-	-	-5.56 (0.0009)***	1.75 (0.1235)
11	77.43 (<0.0001)****	1.12 (0.3069)	-3.36 (0.0151)*	-1.12 (0.3056)	-
12	66.48 (<0.0001)****	2.66 (0.0377)*	-2.95 (0.0256)*	-	0.50 (0.6319)
13	26.38 (0.0007)***	2.86 (0.0287)*	-	-0.31 (0.7697)	0.43 (0.6790)
14	109.19 (<0.0001)****	-	-7.04 (0.0004)****	-3.90 (0.0080)**	2.04 (0.0880)
15	69.85 (0.0001)****	0.34 (0.7478)	-3.88 (0.0117)*	-1.82 (0.1287)	1.47 (0.2015)

* refers to p value ≤ 0.05 , ** refers to p value ≤ 0.01 , *** refers to p value ≤ 0.001 and **** refers to p value ≤ 0.0001 .

The second step is to add another variable to the model. The results when adding inter-particle distance to the model can be seen in Model 5 (Table 4.2 and Table 4.3). The overall model was significant ($p < 0.0001$). Results from Student t -test shows that both inter-particle distance and intersections were significant ($p < 0.05$). Instead of adding inter-particle distance, the sum of particle perimeter was added to the model, as shown in Model 8. This model had a significant p value (< 0.0001) and each variable passed Student t -test, showing that this variable is significant to add to the model. While Model 5 and 8 passed F -test and all parameters in both models passed Student t -test, Model 9, which contains the number of intersections and particle area, shows the insignificant parameter. There was one slope coefficient that its p value was over 0.05 (0.5640), specifically the particle area. Therefore, the particle area was excluded from the model, while the inter-particle distance and sum of the perimeter were possibly put in the model.

The next step is to add more parameter into the model and again re-evaluate each variable using the p value of Student t -test. When adding either the sum of the perimeter to Model 5 or adding inter-particle distance to Model 8, the p value was below 0.0001, showing that this model is statistically significant (Model 11). However, there were two parameters including inter-particle distance and sum of perimeter having its p value over 0.05 (Table 4.3), suggesting that adding more variables are not necessary. This demonstrates that the model having the three independent variables of the ones considered (Model 11) was not suitable for predicting drug release.

There are now only two models to be considered including Model 5 and 8. All statistical indicators were assessed to choose the best model. As shown previously, the criteria include high R^2 or $\bar{R}^2$ and low errors. Both models had the same R^2 and $\bar{R}^2$ indicating the similar performance of the model. However, Model 5 had the smallest error as its MAE was a little lower than that of Model 8 (Table 4.2). The MAE measures the average magnitude of the errors in each regression model. Data in Model 5 and 8 shows that the averaged predicted dissolution values were 2.7983 and 2.8475 % deviated from the experimental

values respectively. It demonstrates that Model 8 had lower precision than Model 5. The multiple linear regression model based on inter-particle distance and intersections (Model 5) then was the best model with the highest predictive power of 96 %.

Observing the residuals from Model 5 as a function of predicted dissolution values at 30 min can be seen in Figure 4.14. In this study, a residual is calculated by subtracting experimental dissolution values from linear regression modelling data. It can be seen that the plot of Model 5 shows highly precise predicted values with the highest error of about 5 % deviated from the experimental dissolution data. This suggests that the linear regression model according to the inter-particle distance of HPMC and number of HPMC intersections provides the greatest predictive power and most accurate outcomes among all models.

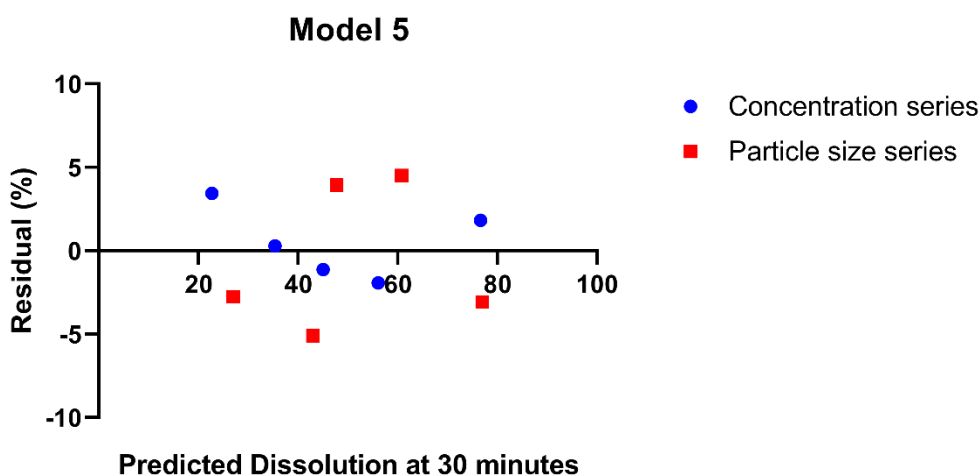


Figure 4.14. Residuals plotted against the predicted dissolution of caffeine at 30 min of Model 5 indicating the data from concentration and particle size series.

To conclude, this work suggests that the distribution of HPMC, represented by inter-particle distance and number of intersections are indicative of dissolution of caffeine at 30 minutes. Additionally, caffeine release appears to be less influenced by HPMC particle characteristics such as particle area, perimeter, length and width.

4.4.5.3 Predicting caffeine dissolution based on the inter-particle distance model

As discussed in Section 4.4.5.1, the inter-particle distance and number of intersections are critically predictive of the release of caffeine from the HPMC matrix tablets with different formulation factors, specifically polymer concentrations and particle size fractions. It is possible to develop a full series of linear regression models in order to predict the cumulative dissolution of caffeine with regard to different dissolution time point up to 60 minutes. Statistical indicators were considered to determine the predictive performance of each mathematical model.

It can be seen in Table 4.4 that the simple linear regression models for predicting the early dissolution percentages of caffeine starting from 4 to 60 min were developed and the corresponding performance indices were calculated to assess their prediction efficiency. It is found that the R^2 and $\bar{R}^2$ were nearly 1 with a small value of errors, indicating a high performance of the models. After ten minutes, R^2 and $\bar{R}^2$ declined as a function of dissolution time, whereas, MAE increased as a function of time. The findings suggest that the models based on the inter-particle distance reduce the quality in predicting the release characteristics of caffeine as a function of dissolution time. The inter-particle distance may significantly impact the early dissolution of caffeine at 8 - 10 min and the release becomes less predictable possibly due to a major change in the HPMC mechanism. Water penetration may predominate initially and progressively change to the mechanism in which drug diffusion and gel erosion dominate. Then the impact of the inter-particle distance would be less significant in the later stage of dissolution.

Table 4.4. Linear regression models to predict the dissolution of caffeine at early stage dissolution.

Time (minutes)	Linear regression model	R^2	$\bar{R}^2$	MAE
4	$y_t = 2.7389x_1 - 0.003128x_2 - 33.6732$	0.8976	0.8841	2.6285
6	$y_t = 3.2942x_1 - 0.01187x_2 - 31.8842$	0.9480	0.9332	2.4722
8	$y_t = 3.3492x_1 - 0.01789x_2 - 25.1195$	0.9574	0.9452	2.3601
10	$y_t = 3.3198x_1 - 0.02286x_2 - 18.2317$	0.9635	0.9530	2.2609
15	$y_t = 3.1381x_1 - 0.03143x_2 - 3.8667$	0.9691	0.9602	2.4067
30	$y_t = 2.6182x_1 - 0.04736x_2 + 27.1719$	0.9696	0.9609	2.7983
45	$y_t = 2.0091x_1 - 0.05913x_2 + 54.7128$	0.9612	0.9501	3.0488
60	$y_t = 1.4140x_1 - 0.06614x_2 + 76.4876$	0.9506	0.9364	3.4661

y_t is caffeine release at t min (%), x_1 is inter-particle distance (μm) and x_2 is intersection / $2.2 \text{ mm} \times 4.1 \text{ mm}$.

Representative cumulative dissolution profiles of caffeine showing the comparison between the observed and the predicted dissolution data from both formulation parameters are depicted in Figure 4.15. In the concentration series (Figure 4.15A), the linear regression can predict the dissolution of caffeine with a maximum deviation of less than 5 %. The longer the dissolution time, the more the discrepancy between experimental and modelling dissolution values observed in the particle size series (Figure 4.15B). In other words, individual tablets have become less predictable in their release characteristics as the dissolution time increased. This is likely explained by the impact of HPMC on gel release. Once a tablet is wet and HPMC transforms from a solid state to a rubbery state. The complete layer of HPMC particles could be developed and followed by the overall delay in dissolution.

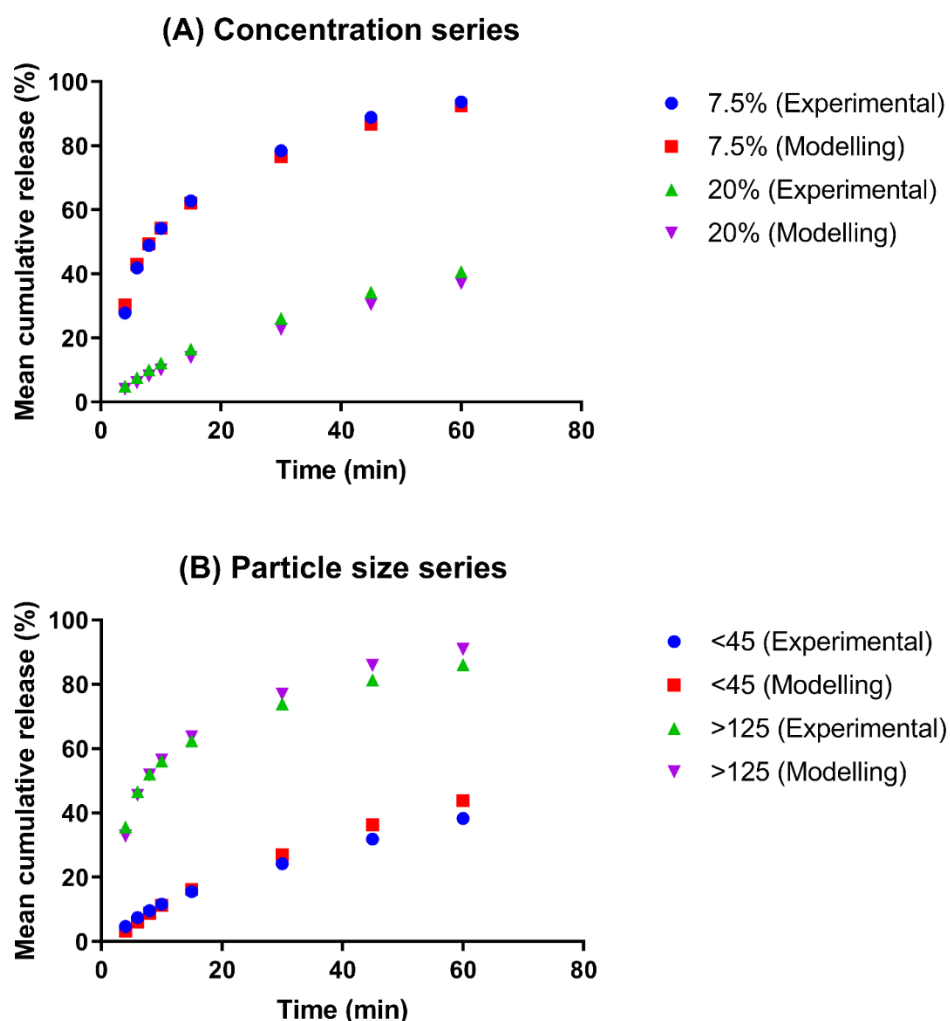


Figure 4.15. Mean cumulative dissolution profiles of caffeine comparing between experimental and mathematical modelling data in concentration and particle size series.

Additionally, the predicted dissolution profiles were compared with the experimental ones using a fit factor, namely similarity factor (f_2). The similarity factor was a parameter from a model independent mathematical approach proposed by Moore (1996)¹⁹⁵ to compare the similarity between dissolution curves of two products. It is a reliable estimate recommended by FDA to assure product sameness between the reference and test product. This parameter was adopted to measure the closeness between the two dissolution profiles in this study which includes the profiles developed by the linear regression model based on inter-particle distance and intersections and the experimental profiles from the dissolution test. The f_2 values in a range between 50 to 100 indicate sameness

or equivalence between the two dissolution profiles. If the f_2 value is 100, the two profiles are identical.

Table 4.5. Similarity factor (f_2) compared between the experimental ($n = 3$) and predicted dissolution profiles ($n = 3$).

Formulation	f_2
7.5%	87.33
12.5%	70.36
15%	76.00
17.5%	99.20
20%	76.89
< 45 μm	76.10
45-63 μm	66.91
63-90 μm	72.97
90-125 μm	63.18
> 125 μm	76.25

What is striking about the values in Table 4.5 is that all f_2 values are over 50. This finding indicates that the predicted dissolution profiles are close to those observed in dissolution testing. However, it is noted that the number of data points is limited to 3 units each. The inter-particle distance and intersection of HPMC reflect the importance of the percolation threshold. This parameter can be used to assess whether or not the matrices would successfully develop an effective surface gel barrier.

4.5 Conclusions

HPMC based matrix tablets with caffeine as a model API were selected for the investigation of a relationship between the formulation variables and drug dissolution. The microstructure of cross-sectioned tablets was investigated to observe the change of HPMC distribution and elucidate HPMC's function regarding the effect of HPMC concentration and particle size. In the concentration series, Raman and ToF-SIMS chemical images demonstrated an increase of HPMC clusters or networks in the 30 %w/w HPMC tablet compared to the 7.5 %w/w HPMC tablet. The networks also were comparatively high in the tablets when reducing particle size fractions, specifically in the size of lower than 45 μm .

The linear correlation between HPMC image parameters obtained from both concentration and particle size series and the early dissolution of caffeine was determined by Pearson correlation coefficients. It is found that the correlation coefficient between the average of the nearest distance between HPMC particles and the dissolution was close to 1, indicating a high correlation between two variables. A mathematical model showing a good correlation with dissolution was successfully developed based on these HPMC parameters to predict the release rate of caffeine at varied dissolution time points. Chemical imaging by ToF-SIMS has successfully provided an insight into the microstructure of tablets and offers a comprehensive analysis of how the formulation factors correlate with drug release. This information is the elucidation of the mechanism behind the capacity of HPMC to control drug release in tablets underpinning long sought after fundamental understandings of such systems. This work could be used to support the further development of pharmaceutical products with tailored release characteristics.

Chapter 5:

Mechanistic understanding toward polymer network formation in governing drug release from hydrophilic matrix tablets

5.1 Introduction

Standard dissolution tests in conjunction with UV spectroscopy/HPLC can determine the level of the drug being released over a given time period. However, an intriguing area in the field of drug delivery and controlled release is the mechanism of drug dissolution. Drug release tests fail to provide insight into a change of tablet's microstructure over time. As previously demonstrated in Chapter 3, ToF-SIMS chemical imaging can be successfully applied to resolve spatial information in a pharmaceutical system, as such could be employed to do so following dissolution.

It is demonstrated in Chapter 4 that the microstructure of matrix tablets in relation to formulation parameters is highly relevant to the physical attributes of the tablets, specifically release performance. Static ToF-SIMS was employed to visualise the chemical distribution of caffeine and excipients along with HPMC connected particles in particular matrix tablet formulations. The present study aims to address other chemical imaging applications of ToF-SIMS to widen current knowledge of the release behaviour of pharmaceutical products. The first part of this chapter (Section 5.4.1) gives an overview of applying dynamic ToF-SIMS to visualise the 3D chemical distribution of hydrophilic matrices. This part focuses upon determination of HPMC interconnectivity. In the latter part (Section 5.4.2) presents an approach using ToF-SIMS to monitor changes in compound distributions when the tablet is exposed to an aqueous medium in the dissolution apparatus. These would broaden the understanding of drug release behaviour from the matrix tablets, especially the HPMC-based formulations.

5.2 Aim and objectives

The aim of this chapter is to provide an insight into drug release mechanisms from HPMC-matrix tablets using ToF-SIMS. The objectives are:

- (1) Optimise experimental parameters for visualisation and depth profiling of HPMC-matrix tablets.
- (2) Visualise the 3D microstructure of caffeine tablets as a function of HPMC concentrations.
- (3) Monitor changes in chemical distribution especially caffeine localisation and HPMC network development during dissolution as a function of HPMC concentrations.
- (4) Monitor changes in chemical distribution especially caffeine localisation and HPMC network development during dissolution as a function of dissolution times.

5.3 Materials and methods

5.3.1 Materials

For Section 5.4.1, caffeine tablets manufactured using a method described in Section 4.3.2 were selected to study the effect of HPMC content on the chemical distribution as a function of depth. Such samples included 7.5 %w/w and 30 %w/w HPMC tablets with a particle size range of less than 125 μm . The tablets are white, round with the diameter of 8 mm and thickness of 4 mm.

For Section 5.4.2, caffeine tablets manufactured using a method described in Section 4.3.2 were selected to observe changes during the dissolution. Such samples included 15 %w/w and 30 %w/w HPMC tablets with a particle size range of 45 – 63 μm . This particle size was constant to address the impact of HPMC level on the microstructure of the tablets during the dissolution procedure.

5.3.2 Preparation of cross-sectioned tablets

The tablets were cross-sectioned as described in Section 0.

5.3.3 Depth profiling and 3D chemical imaging using dynamic ToF-SIMS

For Section 5.4.1, an argon gas cluster ion beam (Ar-GCIB) was used as a sputter source for depth profiling as it can preserve the molecular structure and create quite low damage accumulation compared with other beams, e.g. C_{60}^{+} and monoatomic ion beams. The energy of the sputter beam employed ranged from 10 - 20 keV with target current ranging from 10 – 20 nA. The primary ion beam used for analysis was a 25 keV Bi_3^{+} source which was used to analyse the central area located in the sputtered area. An electron flood gun was employed for charge compensation. The tablet cross-section as prepared using the method described in Section 3.3.2.2 was analysed by the Bi_3^{+} beam over $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ or $250\text{ }\mu\text{m} \times 250\text{ }\mu\text{m}$ areas within the sputtered area of $450\text{ }\mu\text{m} \times 450\text{ }\mu\text{m}$ and $700\text{ }\mu\text{m} \times 700\text{ }\mu\text{m}$. A 256×256 pixel resolution was used. The non-interlaced

dual beam mode (i.e. beams run non-simultaneously) consisted of a continuous cycle of 1 second sputtering and then followed a 0.5 second pause. In the case of the 30 %w/w HPMC tablet with correction, the sample height was adjusted to the original position every 22 minutes. Data acquisition in positive polarity mode and subsequent data processing were acquired using SurfaceLab 6 (ION-TOF GmbH, Münster, Germany). The comparison of ToF-SIMS settings among samples is detailed in Table 5.1.

Table 5.1. The difference in ToF-SIMS settings of each tablet sample.

	30 %w/w	7.5 %w/w	30 %w/w
ToF-SIMS settings	Before	With	With
	corrections	corrections	corrections
FoV ($\mu\text{m} \times \mu\text{m}$)	200×200	200×200	250×250
Crater size ($\mu\text{m} \times \mu\text{m}$)	450×450	700×700	700×700
Energy of Ar-GCIB (keV)	10	20	20
Current (nA)	10	20	20

5.3.4 Optical imaging and profiling

For Section 5.4.1, an optical profilometer (ZDot™, Zeta Instruments, Canada) was utilised to obtain the distance between initial and crater surface due to dynamic SIMS. The optical measurements were carried out using 10 calibration lines with a $5\times$ objective lens (a $3479 \times 2606 \mu\text{m}^2$ field of view) at a scan speed of 1.000 $\mu\text{m}/\text{step}$.

5.3.5 Simulation of *in vitro* release tests

For Section 5.4.2, a USP II (paddle) apparatus (Dissolution Tester DIS 8000, Copley Scientific, UK) was employed to monitor the effect of dissolution time and HPMC concentrations on the microstructure of matrix tablets. Dissolution tests were undertaken in 900 mL de-gassed, de-ionised water at $37 \pm 0.5^\circ\text{C}$. Paddles were rotated at 50 RPM for specified periods, including 5, 15 and 30 minutes. The dissolution tests were stopped after the specified times. Solution

sampling was undertaken for 10 ml to quantify the amount of caffeine. The absorbance values of the collected sample solutions at 273 nm were later measured, using a Cary 50 UV-Vis spectrophotometer system (Agilent, USA). The tablet was transferred to a clean plastic Petri dish using a clean pair of forceps. The hydrated tablet was kept at room temperature overnight to dry.

5.3.6 Microstructural analysis using static ToF-SIMS

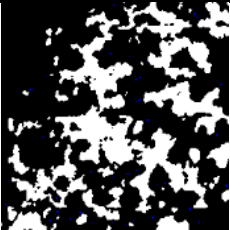
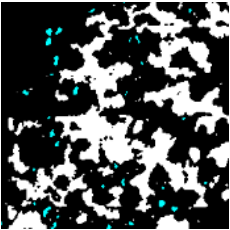
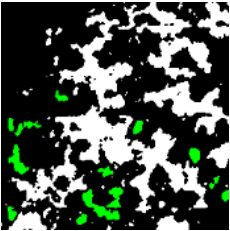
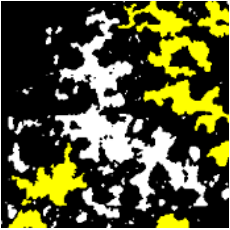
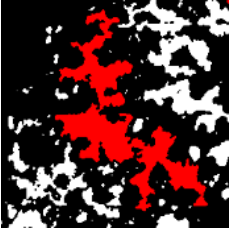
For Section 5.4.2, the visualisation of the chemical distribution of tablet components was carried out using a ToF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany). Characteristic secondary ions of caffeine, HPMC and lactose were identified and chosen, as previously mentioned in Section 3.4.4.1. The tablet samples were analysed using a 25 keV Bi_3^+ primary ion source, rastered over a whole cross-sectioned plane of the tablets. The dimension of the analysed area ranges from 4.0 to 4.5 mm in the x-axis and 7.0 to 7.5 mm in the y-axis, depending on the actual tablet size. The total primary ion beam dose for each analysed area was kept below 1×10^{12} ions/cm² to ensure the static conditions. The compensation of surface charge was carried out by using an electron flood gun to provide low-energy electrons (20 eV). Data acquisition was in a positive polarity mode, as previously discussed in Section 3.4.4.1. Data processing was acquired using SurfaceLab 6 (ION-TOF GmbH, Münster, Germany).

5.3.7 Image analysis

For Section 5.4.2, ImageJ 1.52a software (National Institutes of Health, USA) was applied to differentiate the size of the HPMC particle and identify the network of HPMC. A resolution of 0.250 μm per pixel was set for binary images converting from ToF-SIMS chemical images. The colour threshold was adjusted to justify the boundary of each particle. Then, chemical maps of HPMC were converted into binary images demonstrating HPMC in white colour (colour value of 255). A median filter was applied for the purpose of removing noises using the 'Despeckle' command. The median filter works by replacing each pixel with the median value in its 3 x 3 neighbourhood. The threshold of this

study was set at $10\ \mu\text{m}^2$ meaning that any particles below $10\ \mu\text{m}^2$ are not shown. The microstructural features of the hydrophilic matrices as a function of the period of the dissolution test were determined by means of labelling HPMC particles with different colours according to their area. The characteristics of HPMC particles were then categorised into 5 groups as shown in Table 5.2, including HPMC fine particles (blue), small particles (cyan), large particles (green), attached particles/clusters (yellow) and networks (red).

Table 5.2. Colour assigning showing different groups of HPMC characteristics and particle sizes.

Colour group	HPMC characteristic	Particle size (μm^2)	Example (1000×1000 μm^2)
Blue	Fine particle	$1 \times 10^1 - 1 \times 10^2$	
Cyan	Small particle	$1 \times 10^2 - 1 \times 10^3$	
Green	Large particle	$1 \times 10^3 - 1 \times 10^4$	
Yellow	Attached particle/cluster	$1 \times 10^4 - 1 \times 10^5$	
Red	Network	$1 \times 10^5 - \infty$	

5.4 Results and discussion

5.4.1 3D chemical characterisation of hydrophilic matrix tablets

The correlation between the inter-particle distance of HPMC particles and the dissolution rate of caffeine from hydrophilic matrices is discussed based upon 2D image analysis as shown in Chapter 4. However, 2D imaging does not perfectly illustrate the actual 3D structure of the tablet samples. It is postulated that 3D imaging might represent a point at which interconnectivity of the HPMC particles is established. Until now, there has been no known evidence to confirm the existence of the 3D hydrophilic network structures of HPMC in the hydrophilic matrices (Section 1.3.3.5). In order to overcome the limitations of the 2D analysis of HPMC-based matrix tablets, dynamic ToF-SIMS was employed to investigate the distribution of HPMC particles as a function of depth.

5.4.1.1 3D chemical characterisation of hydrophilic matrix tablets using dynamic ToF-SIMS

The 30 %w/w HPMC tablet with caffeine was used as a representative example for an illustration of the HPMC networks using 3D chemical imaging. As mentioned in Chapter 1, Section 1.2.5.2, the HPMC content of 30 %w/w was recommended by a manufacturer¹⁶ to ensure that the gelatinous layers are completely developed during dissolution. The tablet was cross-sectioned using a microtoming approach as described in Section 0. After the sputter crater of the sample was produced, the area within the crater was analysed using dynamic SIMS (Section 5.3.3) and the depth of the crater was measured using an optical profilometer (Section 5.3.4). The resulting optical profilometry image and profile are shown in Figure 5.1A and B respectively. Using 10 calibration lines, the calculated depth of crater was $220.77 \pm 25.43 \mu\text{m}$. The measurement of depth was then correlated to the acquired sputter times. The average sputter rate ($\mu\text{m/s}$) calculated from the sputtered depth and time was 0.006224. However, the crater was not flat as shown in the optical profile of the 30 %w/w tablet (Figure 5.1B). A reasonable explanation for this might be that the incident angle of the

sputtering cluster beam was obscured by the surface of the tablet itself, as indicated by the blue boxes. As can be observed in Figure 5.1B, the area near the edge of the crater was lost indicating that the sample was unintentionally removed by the sputter beam. In this case, the crater size of $450\ \mu\text{m} \times 450\ \mu\text{m}$ was not enough for the analysed area of $200\ \mu\text{m} \times 200\ \mu\text{m}$. The results thus need to be interpreted with caution and require effective corrections.

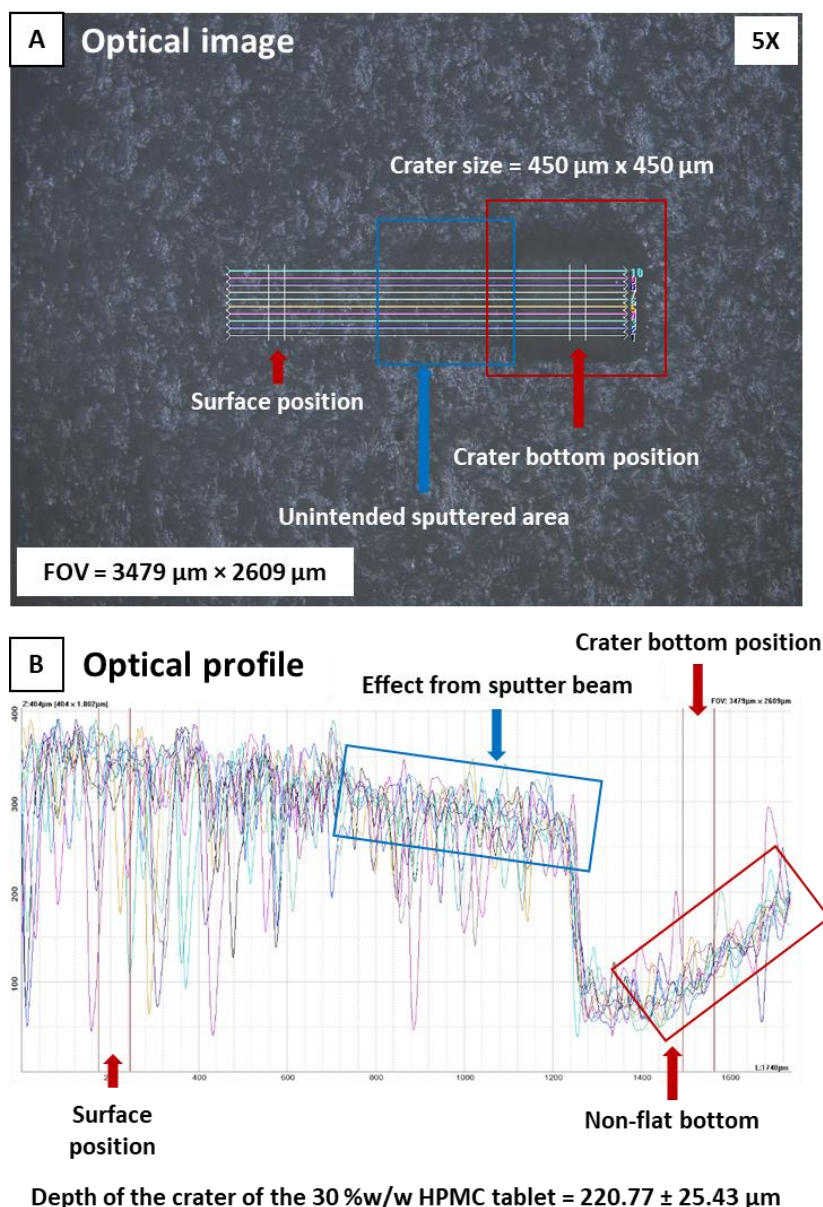


Figure 5.1. Optical profilometry data of the 30 %w/w HPMC tablet cross-section: (A) optical image of the sputter crater and (B) optical profile. Surface and crater bottom positions are indicated.

ToF-SIMS data demonstrating the depth profile and 3D structure of the caffeine tablet with 30 %w/w HPMC can be seen in Figure 5.2A and B respectively. The depth profile (Figure 5.2A) shows the normalised ion intensity of components as a function of the sputter time and measured depth. The intensity of $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$, CH_5O^+ and $\text{C}_{12}\text{H}_{22}\text{O}_{11}\text{Na}^+$, which represents caffeine, HPMC and lactose respectively, varied as a function of depth in the analysed region. The normalised ion intensity of $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$ increased until it reached a peak at the depth of 40 μm . At the same amount of depth, it can be seen that the ion intensity of CH_5O^+ and $\text{C}_{12}\text{H}_{22}\text{O}_{11}\text{Na}^+$ was lower. This demonstrates the presence of caffeine and a lack of HPMC and lactose. Then, the normalised ion intensity of caffeine considerably declined towards the end of the analysis. The peaks of the intensity of HPMC and lactose were at 50 and 100 μm respectively. The difference in ion intensity of all components shows that there were differences in component localisation. This demonstrates the nature of the matrix tablet which is a mixed system. As can be seen in Figure 5.2B and C, it seems that HPMC particles coalesced with each other, forming an interconnected HPMC distribution network throughout the whole analysis region. However, it can be observed that the normalised intensity of all components reduced significantly below 100 μm and the ToF-SIMS data completely disappeared at the end of the analysis (Figure 5.2A). Figure 5.2B and C also illustrate that there was a loss of ToF-SIMS imaging datasets. These problems were probably due to image shifting (Section 2.2.9.5) which normally occurs when depth profiling to great depth. The increasing depth of analysis and the fixed geometry of the analysis beam result in a lateral shift of the analysis position and resulting chemical image. This issue may lead to the reduced intensity and significant loss of ToF-SIMS data found in Figure 5.2. These findings were greatly affected by the limited size of the crater and image shifting. The correction of depth profiling and 3D chemical imaging are then required and discussed in Section 5.4.1.2.

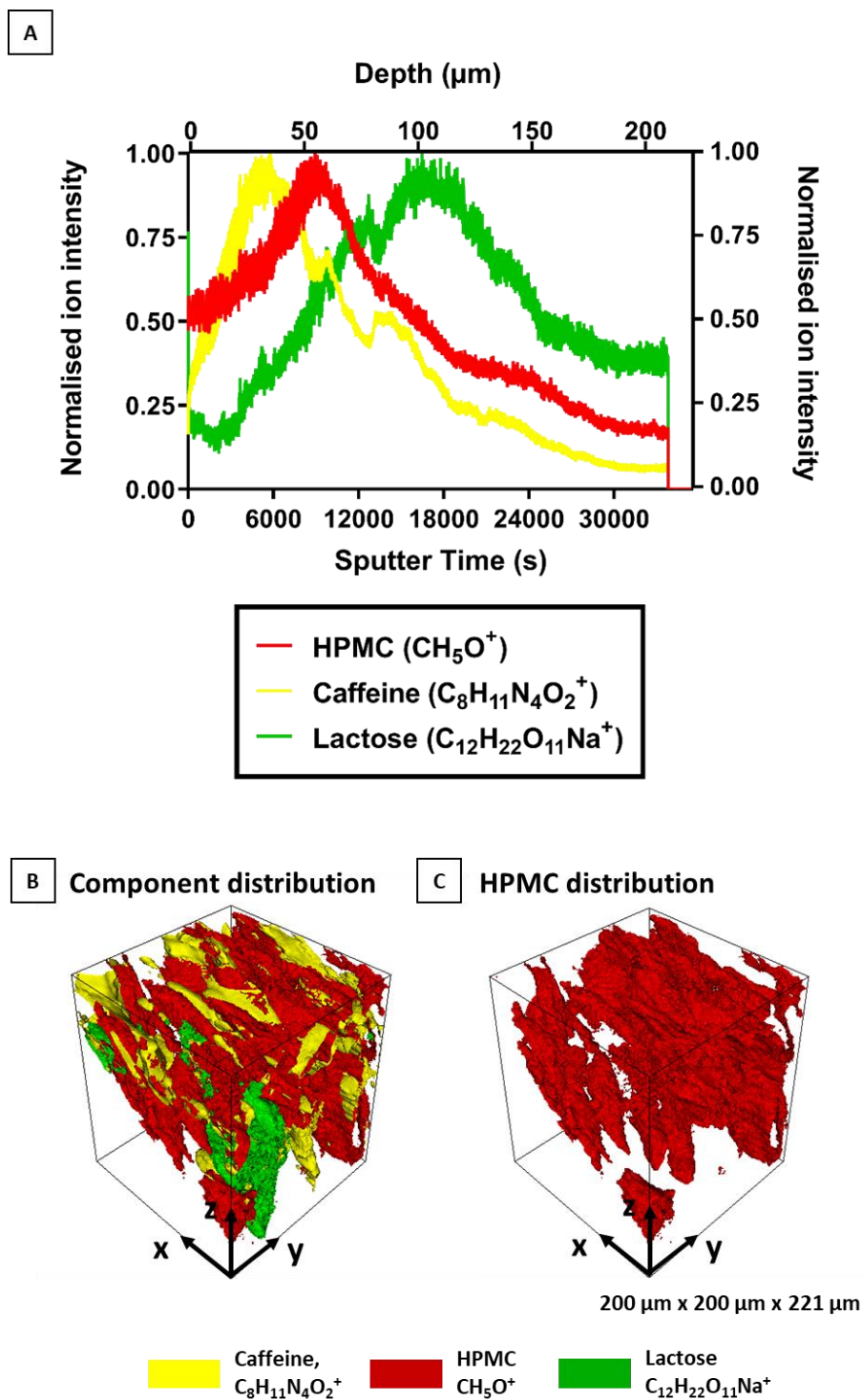


Figure 5.2. ToF-SIMS data of the 30 %w/w HPMC tablet cross-section (A) Depth profile of normalised secondary ions representing HPMC, caffeine and lactose, (B) 3D chemical imaging of components in the caffeine tablet and (C) distribution of HPMC. Data taken from the round tablet with the diameter of 8 mm and thickness of 4 mm.

5.4.1.2 Corrections of image shifting in 3D chemical characterisation of hydrophilic matrix tablets using dynamic ToF-SIMS

In order to avoid the non-flat base of the sputter crater found in Section 5.4.1.1, the size of the sample's crater needs to be much wider than the analytical area of interest. This would avoid the edge of the crater being sputtered by the GCIB which may finally lead to the incomplete removal of the substrate during the sputtering process. This approach was then employed in the analysis of a 7.5 %w/w HPMC tablet. The sputter crater was significantly enlarged from $450\text{ }\mu\text{m} \times 450\text{ }\mu\text{m}$ to $700\text{ }\mu\text{m} \times 700\text{ }\mu\text{m}$. However, increasing the size of the sputter region would result in a longer acquisition time. In order to avoid a considerable amount of analysis time, the energy and target current of the Ar-GCIB were enhanced from 10 to 20 keV and from 10 to 20 nA respectively.

The chemical distribution of the 7.5 %w/w HPMC tablet as a function of depth was acquired. The sample was profiled and the depth of created sputter crater was measured using optical profilometry. As shown in the optical profile of the 7.5 %w/w HPMC tablet (Figure 5.3), the bottom of the crater was found to be relatively flat due to changing in the size of the crater. The calculated depth of the crater was $182.42 \pm 39.03\text{ }\mu\text{m}$ with the sputter rate ($\mu\text{m/s}$) of 0.01868. The calculated sputter rate was triple the previous 30 %w/w HPMC sample (Section 5.4.1.1) as a direct result of the higher beam dose per area. Changes in tablet composition may also slightly change the sputter rate but these effects could only be calculated with an assessment of each individual component.

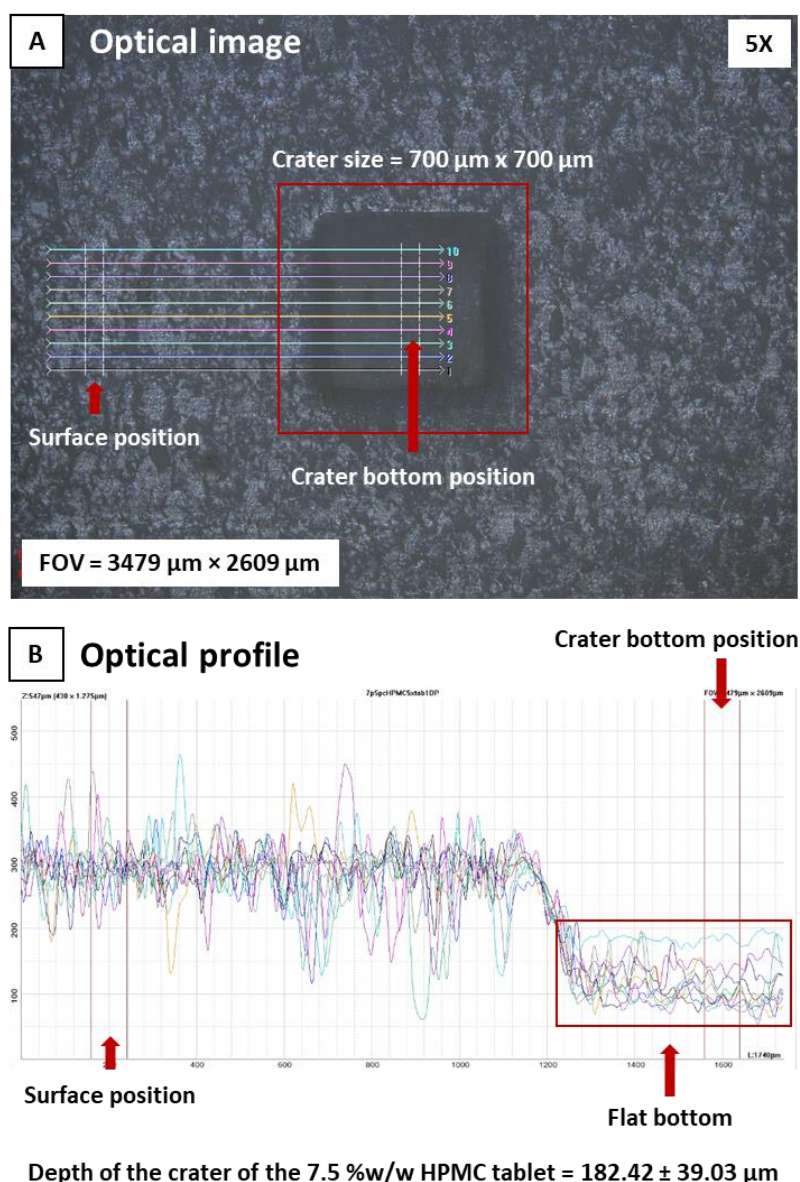


Figure 5.3. Optical profilometry data of the 7.5 %w/w HPMC tablet cross-section showing (A) optical image of the sputter crater and (B) optical profile. Surface and crater bottom positions are indicated.

The depth profile showing the distribution of characteristic ions of each raw material found in the 7.5 %w/w HPMC tablet is displayed in Figure 5.4A. The normalised ion intensity peaks of caffeine, HPMC and lactose were at different depths. It highlights that this system was multicomponent consisting of a number of materials. However, this depth profile is not the main focus in this section. The more important aspect of this experiment is to determine the level of the interconnectivity of the polymer in the tablets.

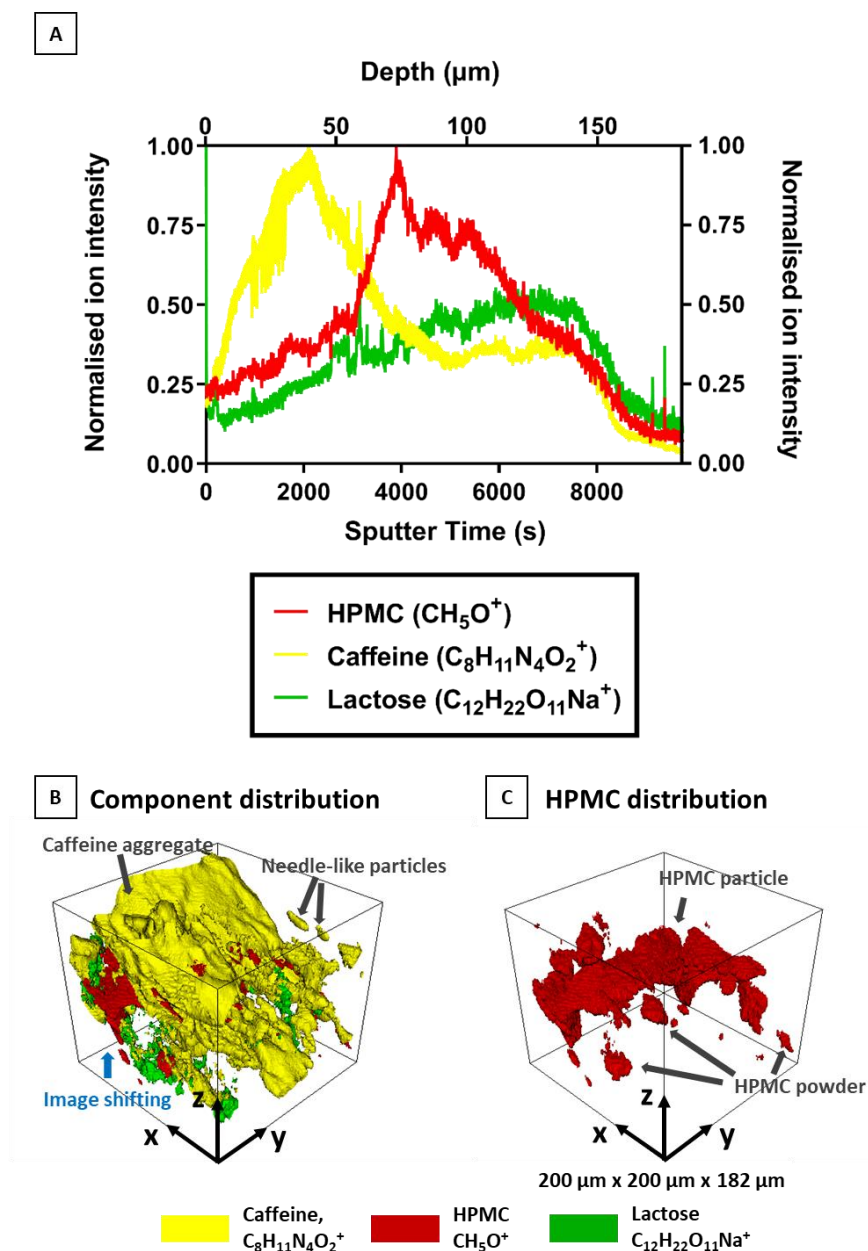


Figure 5.4. ToF-SIMS data of the 7.5 %w/w HPMC tablet cross-section (A) Depth profile of normalised secondary ions representing HPMC, caffeine and lactose, (B) 3D chemical imaging of components in the caffeine tablet and (C) distribution of HPMC. Data taken from the round tablet with the diameter of 8 mm and thickness of 4 mm.

Examination of the ToF-SIMS secondary ion images of 7.5 %w/w HPMC tablets as a function of depth can be seen in Figure 5.4B. This data demonstrates the localised distribution of caffeine. It is clearly seen that caffeine is the most prominent material. Individual needle-shaped particles and their aggregates were identified and are shown in Figure 5.4B. This finding is in accord with SEM

(Section 3.4.1) and static SIMS data (Section 4.4.2) that there may be a lump of caffeine exhibited. Discrete HPMC particles can be observed as this formulation contains the low content of HPMC. There were two forms of HPMC found. The first form was globular HPMC powders, while another one was long fibrous HPMC particles. There was only one long fibrous particle that can be detected in the analysis area. This could occur as the particle size of HPMC is expected to be less than 125 μm . The finding that the long particulate HPMC particles were rarely found in this area is in agreement with the static SIMS data. As reported in the 2D data reported in Section 4.4.2, this tablet formulation shows the large inter-particle distance of HPMC.

The size of the sputter region was increased and this appears to have the improvement in removing the sample materials and collecting ToF-SIMS data. The most striking observation to emerge from the comparison of the 3D chemical images between this and the previous datasets (Section 5.4.1.1) was that the image data was collected until the depth of 180 μm with a small lateral shift observed. The geometry of the primary ion beams and the position of the sample surface may still lead to the shifting of the focal position and the secondary ion images leading to a distortion of the 3D rendered structures.

A second modification of the experimental details was applied in the form of manually adjusting the sample height during ToF-SIMS data acquisition (Section 5.3.3). This approach was undertaken with the 30 %w/w HPMC tablet. After this manual adjustment of the sample height, the step measurements using optical profilometer was made and the result can be seen in Figure 5.5. As shown in the optical profile of the 30 %w/w HPMC tablet (Figure 5.5A), a flat crater base can be observed as a result of having a large sputter area (700 $\mu\text{m} \times 700 \mu\text{m}$). The calculated depth of the crater was $93.48 \pm 14.39 \mu\text{m}$ with the sputter rate ($\mu\text{m/s}$) of 0.03124.

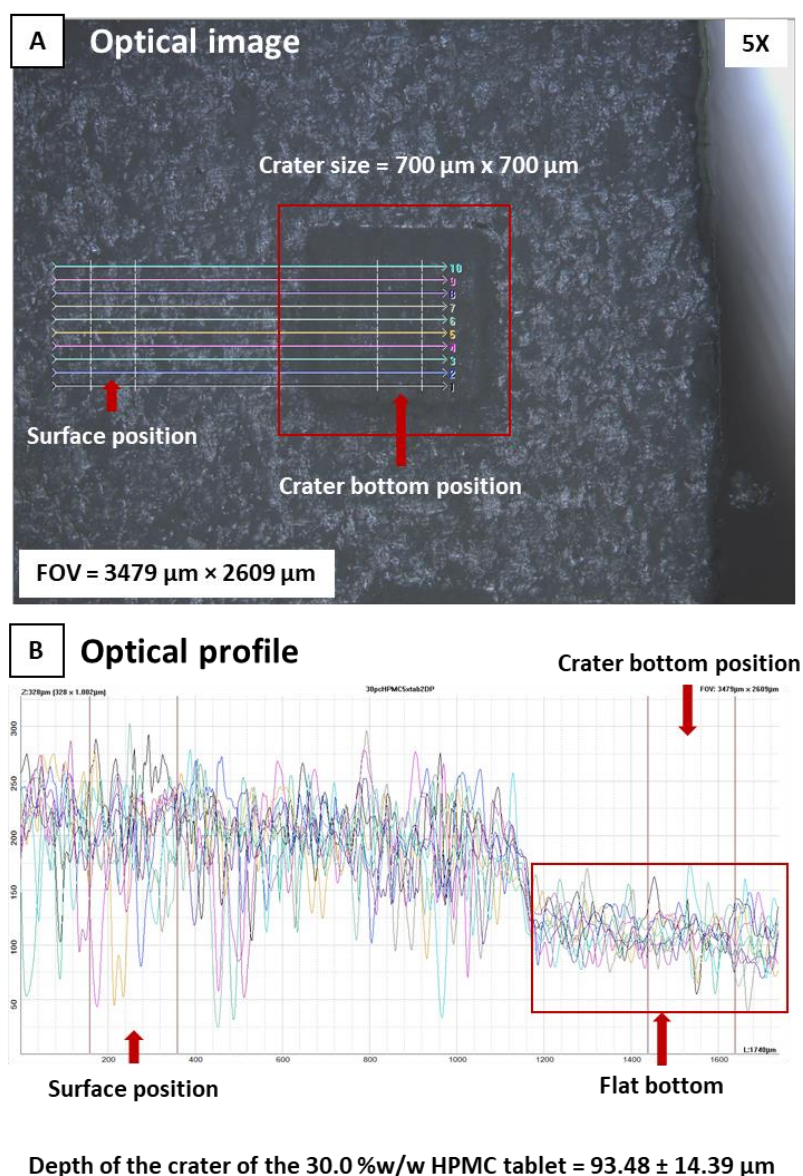


Figure 5.5. Optical profilometry data of the 30 %w/w HPMC tablet cross-section after manual stage adjustment showing (A) optical image of the sputter crater and (B) optical profile.

Surface and crater bottom positions are indicated.

As illustrated in Figure 5.6A, the characteristic ions of HPMC and lactose remained stable confirming that the presence of HPMC and lactose were constant throughout the sputtering process. However, there was the presence of caffeine at the depth between 50 to 75 μm , as evidenced by the highest normalised ion intensity of $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$. The depth profile of this tablet demonstrates the distribution of the chemicals in the matrix tablets where a randomly homogeneous mix of components is expected.

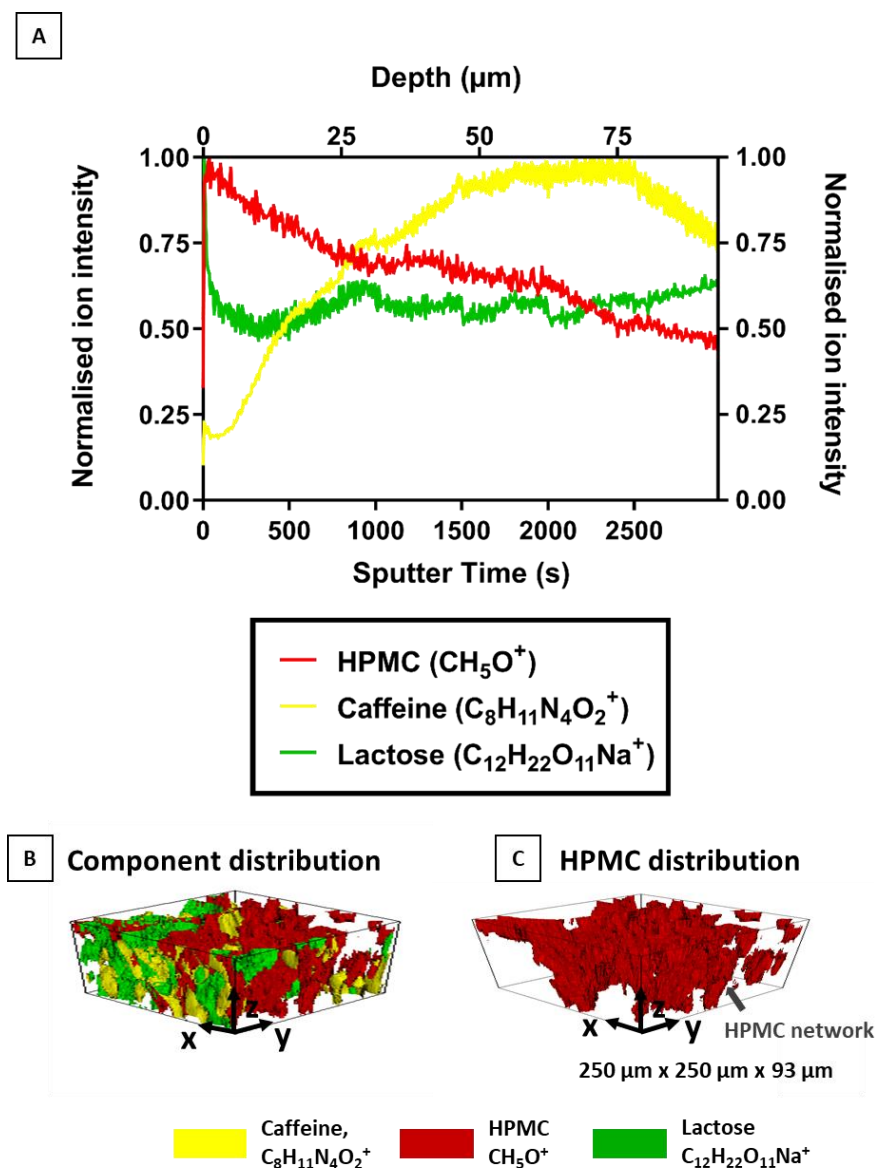


Figure 5.6. ToF-SIMS data of the 30 %w/w HPMC tablet cross-section after stage adjustment: (A) Depth profile of normalised secondary ions representing HPMC, caffeine and lactose, (B) 3D chemical imaging of components in the caffeine tablet and (C) distribution of HPMC. Data taken from the round tablet with the diameter of 8 mm and thickness of 4 mm.

As shown in Figure 5.6B and C, the 30 %w/w HPMC tablet clearly illustrates the interconnectivity of the HPMC particles. Compared to the 7.5 %w/w HPMC tablet, the HPMC particles were more prevalent in the 30 %w/w HPMC and the HPMC networks were relatively observed rather than individual particles. This observation is in line with percolation theory in which the HPMC particles already formed infinite clusters at 30 %w/w HPMC. Additionally, this evidence can support the recommendation by a manufacturer and the patent that 30 %w/w

of HPMC is required for controlled release (Section 1.2.5.2). The network of polymer could affect the diffusion, gelation and erosion properties of the polymers during hydration. It is, therefore, likely that such connections delay the release of caffeine. The 3D chemical image of this sample confirms that the method in which the sputter region was enlarged and sample height was adjusted as equal amount as the sputtered depth enabled accurate representation of tablet microstructure. This modification can compensate the focal changes of the primary beam associated with the removal of the materials and optimise the height to accommodate the lateral shifting. Therefore, it allows the determination of the HPMC interconnectivity.

5.4.2 Microstructural changes of hydrophilic matrix tablets after dissolution

In an attempt to monitor if the microstructure of tablets and the spatial distribution of the species of interest will change over specific dissolution periods, dissolution tests were performed using the method stated in Section 5.3.5. The microstructure of the tablets after the dissolution test was obtained and compared with the tablet which had not undertaken the dissolution test (0 min) as discussed in Section 5.3.6. In order to confirm that the distribution of HPMC particles can affect the release of caffeine during the dissolution, the localisation of caffeine and HPMC was overlaid using ToF-SIMS in conjunction with image analysis as discussed in Section 5.3.7.

5.4.2.1 Effect of dissolution time on component distribution of cross-sectioned tablets

Figure 5.7 compares the chemical images of the pre and post dissolution tablets with 15 %w/w HPMC. The tablets experienced different dissolution times, including 5, 15 and 30 minutes. The chemical images shown include the total, HPMC and caffeine ions (CH_5O^+ and $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$ respectively). The most remarkable finding from Figure 5.7 is that the $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$ ion images (Figure 5.7C) of all post dissolution tablet samples demonstrated substantially different distributions of caffeine from the pre dissolution tablet. At 0 min of the

dissolution test, the caffeine particles and clusters can be seen (Figure 5.7CI). They were uniformly distributed across the cross-sectional plane of the tablet. After the dissolution for 5, 15 or 30 minutes, the distributions of caffeine were no longer homogeneous (Figure 5.7CII - IV). The number of caffeine clusters drastically reduced after the dissolution. The caffeine particles varied spatially across the plane in the forms of individually particle clusters and rather indistinct signals in the background. The huge loss of caffeine particles could be explained by that they were already released due to dissolution.

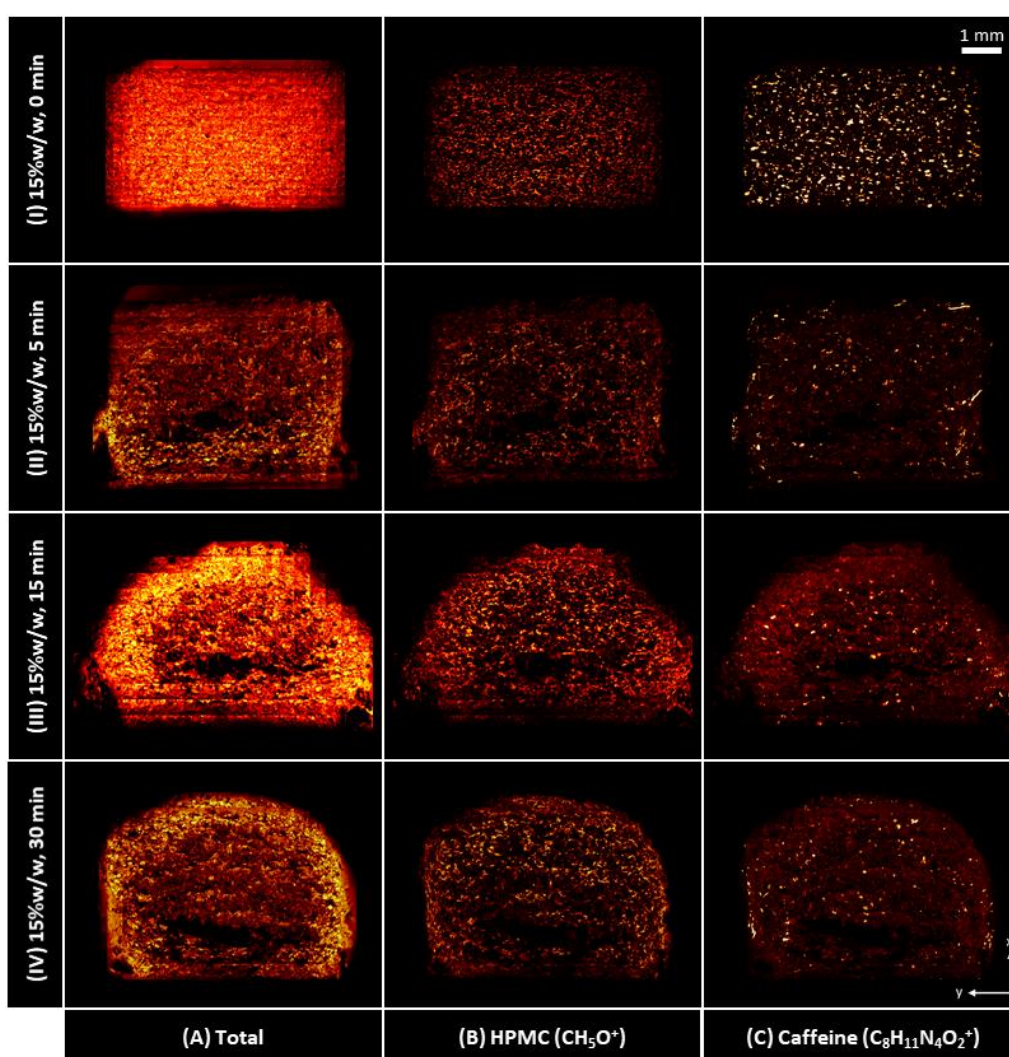


Figure 5.7. ToF-SIMS images including (A) total ion intensity, (B) HPMC and (C) caffeine images comparing the differences of tablet microstructure between different time points, 0, 5, 15 and 30 min of the 15 %w/w HPMC tablet.

The CH_5O^+ ion which was indicative of the distribution of HPMC can be observed in Figure 5.7B. In the pre dissolution sample (Figure 5.7BI), the distribution of HPMC particles was fairly uniform with particle attachments somewhat observed. The distribution of HPMC in all post dissolution tablets (Figure 5.7BII - IV) was fairly similar to the pre dissolution tablet. However, it can be observed that there were more HPMC clusters and networks which seemed to locate near the edge of the post dissolution samples compared to the 0 min dissolution data.

It can be seen from the total ion images of Figure 5.7A that there is a lack of ToF-SIMS data in all post dissolution tablets correlating to a small fracture on the cross-sectioned surface of the tablets. These cracks occurred following immersion of the tablets in an aqueous medium which causes partial disintegration and dissolution of the drug in the tablets. The partially dissolved tablets lose their compactibility upon water hydration, thus demonstrating cracks when they were embedded using resin during sample preparation. The example of the post dissolution tablet at 30 minutes with the small crack near the base of the cross-sectioned tablet can be seen in Figure 5.8. It should be noted that the cracks slightly affect the ToF-SIMS analysis (Figure 5.7IV), especially data in the crack. However, as these affected areas are apparent in the total ion images, they can be readily discounted in the analysis.



Figure 5.8. Representative hydrated tablet with a small crack near the base of the cross-section.

In addition to the ToF-SIMS images, the differences of $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$ (caffeine) and CH_5O^+ (HPMC) normalised intensity were also examined. As shown in Figure 5.9, there was a noticeable reduction in the $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$ ion intensities of

the dissolution point at 5, 15 and 30 minutes when compared to 0 minute. This data is in agreement with the ToF-SIMS images shown in Figure 5.7CII - IV. While a substantial loss of caffeine observed, there was a little increase of the CH_5O^+ ion intensity between the tablet pre and post dissolution testing. The normalised CH_5O^+ ion intensities compared amongst all dissolution time points were approximately similar at 2×10^{-3} .

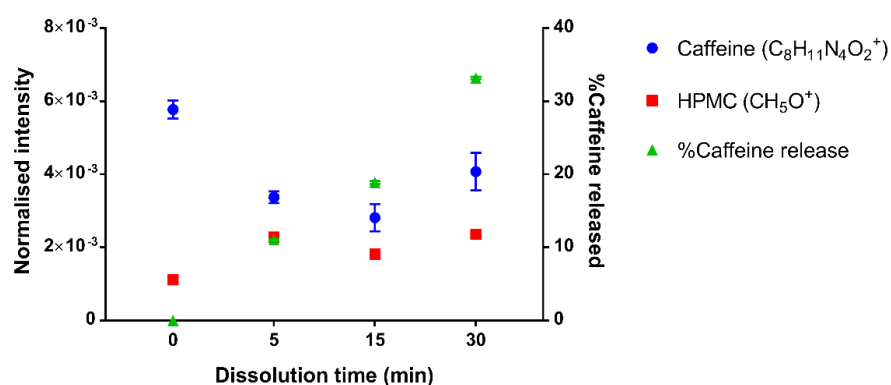


Figure 5.9. ToF-SIMS ion intensity data (left y-axis) highlighting the differences in caffeine ($\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$) and HPMC (CH_5O^+) as a function of dissolution time points with reported caffeine dissolution (right y-axis).

The changes in the component distribution of chemicals with regards to dissolution times can be discussed as follows. After 5 min of dissolution, caffeine may be partially dissolved due to water penetration into the tablets as evidenced by the reduced number (Figure 5.7C) and normalised intensity of caffeine (Figure 5.9). The normalised intensity of CH_5O^+ ion, which represents HPMC particle distributions, was slightly increased after the dissolution demonstrating that the HPMC particles were swelled as a result of the dissolution medium. This corresponds with the swellable properties of HPMC after exposure to water. This attachment of HPMC particles will be demonstrated and discussed in more details in Section 5.4.2.2. Ideally, the complete gel barrier should be formed from HPMC attachment, thus delaying the release of an API. The apparent loss of $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_2^+$ ion intensities (Figure 5.9) demonstrates that the drug particles were still extensively released at the longer dissolution time (15 and 30 minutes) even though there was partial gelation of HPMC particles. The caffeine release of about 20 % and 30 % shown in Figure 5.9 was found at

15 and 30 minutes respectively. Increased caffeine release when it was 30 minutes may be that the results were from two different tablets. It is assumed that the level of HPMC (15 %w/w) may not be sufficient to create a complete barrier to water penetration resulting in the deviation in caffeine release. The constant normalised ion intensities of HPMC (CH_5O^+) at 2×10^{-3} were observed after undertaking the dissolution tests (Figure 5.9). This finding may be as a result of the short dissolution time (no longer than 30 minutes) used in this study where the erosion of the HPMC gel layer might not have occurred.

5.4.2.2 Effect of dissolution time on formation of HPMC networks using image analysis

It is hypothesized that the distribution of HPMC in the matrices may be responsible for the localisation of the caffeine particles. Compared to optical imaging techniques and MRI which are less chemically specific, ToF-SIMS can give a better understanding of the component distribution of tablet components. Image analysis on the ion images of the 15 %w/w HPMC tablets using ImageJ was performed in order to determine where caffeine was located after the dissolution in relation to the distribution of HPMC. The HPMC particles were labelled with an artificial colour scale highlighting the difference in size of each HPMC particle (Section 5.3.7). The locations at which the interconnected particles formed were identified and the co-localisation between caffeine and HPMC was assessed.

The colour coded maps of the pre dissolution tablet in comparison to the hydrated tablets are illustrated in Figure 5.10. With the form of mapping (Section 5.3.7), the connections between neighbouring HPMC particles can be demonstrated with warm colours (yellow and red). Figure 5.10AI shows that there were no HPMC networks (as labelled in red) on the map of the pre dissolution sample. There were progressively higher particles labelled with warm colour when increasing the dissolution time. Connected HPMC particles in the forms of clusters or networks were generally found especially near the tablet's surface after 15 minutes dissolution (Figure 5.10AIII). Caffeine particles seem to co-localise mainly with the red HPMC clusters (Figure 5.10BIII).

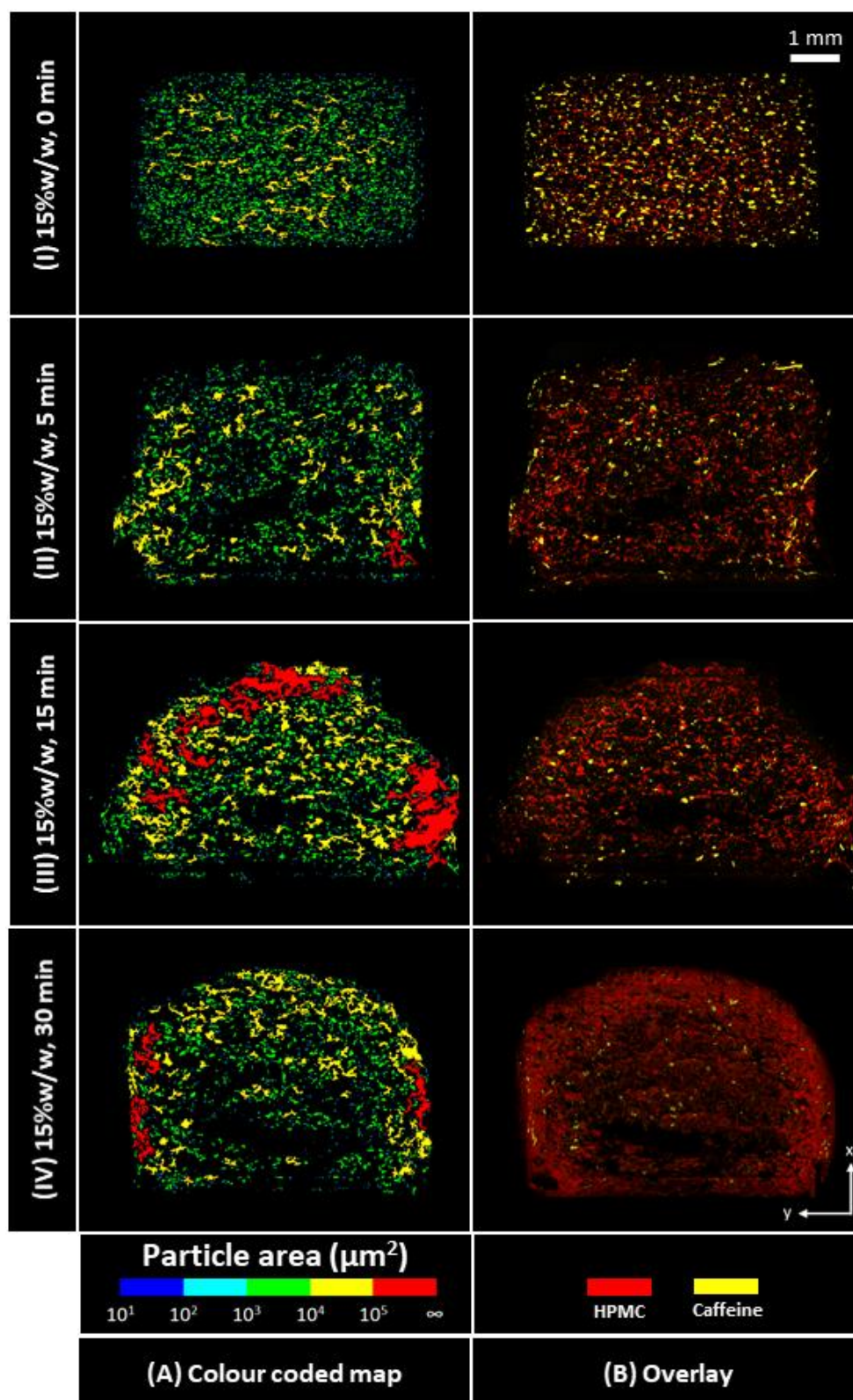


Figure 5.10. (A) Colour-labelled maps of HPMC particles of the 15 %w/w HPMC tablet as a function of dissolution time showing different localisation of HPMC particles depending on HPMC particle area (as displayed as magenta, blue, cyan, green, yellow and red) and (B) HPMC and caffeine overlay.

In all samples, the most prominent HPMC particles were fine particles (blue) (Figure 5.11A). However, when the total particle area from each colour was measured (Figure 5.11B), it can be observed that the large particle (green) was accounted for the largest particle area at 0 and 5 minutes dissolution. After 15 minutes, the HPMC cluster was the highest proportion. This demonstrates that the HPMC particles were not totally interconnected in 0 and 5 minutes, whereas, there were more HPMC clusters after 15 minutes dissolution.

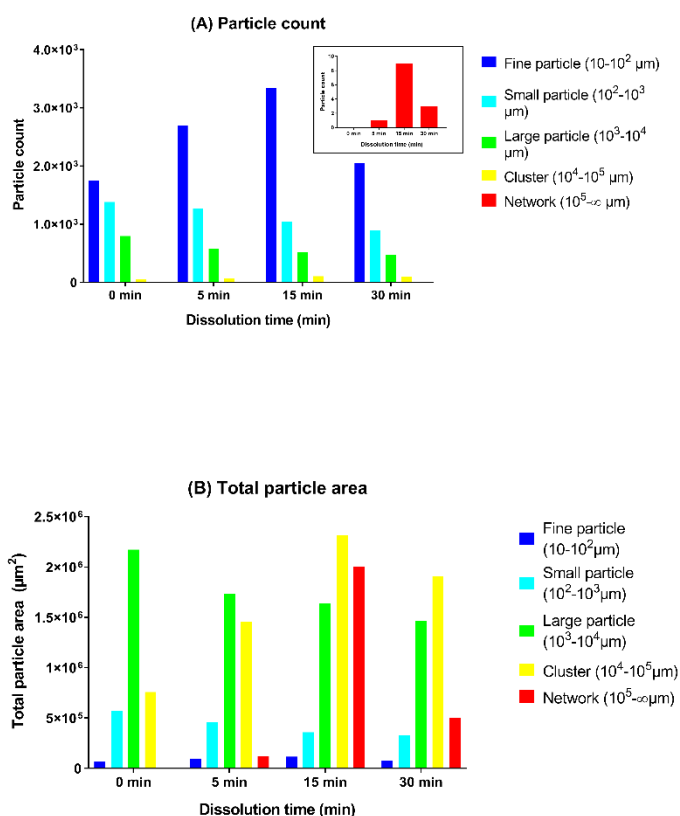


Figure 5.11. Particle count and total particle area of HPMC particles of the 15 %w/w HPMC tablets based on their characteristics at different dissolution time points.

Image analysis demonstrates the changes in five characteristics of HPMC particles after dissolution in relation to the distribution of caffeine. When the tablet came into contact with an aqueous medium, the HPMC started to swell. This resulted in marked increases in the total area of connected HPMC particles and networks displayed in yellow and red and slight decreases in the total area of individual HPMC particles with longer dissolution time (Figure 5.11B). However, the concentration of HPMC of 15 %w/w may not be enough to form

a complete HPMC layer at the outer layer of the tablet after dissolution. When the complete HPMC layer was not developed, it is not possible for these particular matrices to retain the caffeine particles in the centre of the tablet. It can be seen that the heterogeneous distribution of caffeine was observed (Figure 5.10BII). After the hydration of the tablet for 15 minutes, HPMC clusters were developed along with a considerable number of HPMC fine particles. The spatial distribution of caffeine within the tablet may possibly be related to the interconnected HPMC areas as caffeine particles were found largely in the HPMC networks.

One of the major limitations of this experiment is that it is not a time-lapse study of one tablet being monitored. The tablets used for each specified time point in this study might be slightly different in size. It can also be noted that the cross-sectional plane of each tablet may not be the same among these tablets. Owing to the drawbacks associated with ToF-SIMS, the tablets were sampled from the same batch to reduce the difference amongst each tablet. Another limitation is that the samples were analysed in a vacuum chamber during ToF-SIMS analysis and as such must be in a dry state. However, the cross-sectioned data is representative of the component distribution of the tablets at each dissolution time point.

5.4.2.3 Effect of polymer concentration on component distribution of cross-sectioned tablets

The simulation of the dissolution test was conducted for caffeine tablets with 30 %w/w HPMC to compare the effect of HPMC concentrations on the microstructure of HPMC matrix tablets containing caffeine. The 15 minutes dissolution time was selected based on the previous results (Section 5.4.2.2) showing the effect of HPMC spatial distribution on the co-localisation of caffeine particles. Figure 5.12 compares the chemical images of the cross-sectioned caffeine tablets prepared with 30 %w/w HPMC pre and post dissolution for 15 minutes. In this case, an artefact was found in the total ion (Figure 5.12AII) and ion images (Figure 5.12BII and CII) of the post dissolution tablet with regard to a small fracture on the cross-sectioned surface of the tablets.

The location of this fracture was at the bottom of the tablet which has a subtle interference on the analysis which could be ignored in the analysis.

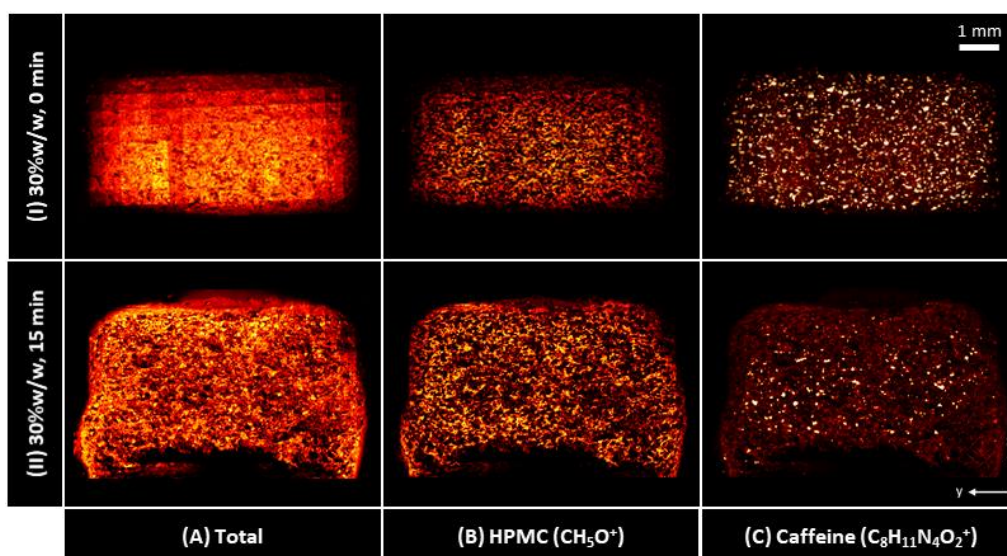


Figure 5.12. ToF-SIMS images including (A) total ion intensity, (B) HPMC and (C) caffeine images comparing the differences of tablet microstructure between different time points, 0 and 15 minutes of the 30 %w/w HPMC tablet.

At 0 minutes dissolution the caffeine particles in the cross-sectioned tablet seemed to be homogeneously distributed (Figure 5.12CI). After the tablet underwent dissolution testing, the distribution of caffeine particles was non-homogeneous and significantly different from the pre dissolution data, as shown in Figure 5.12CII. There was a significant reduction of caffeine particles on the outer edge of the cross-sectioned tablet with localisation in the centre area (Figure 5.12CII). When comparing the 30 %w/w HPMC tablet data with the 15 %w/w HPMC tablet after the dissolution test for 15 minutes (Figure 5.7III), it is clearly seen that the patterns of caffeine distribution between the two formulations are very different as the 15 %w/w HPMC formulation exhibited the non-uniform distribution of caffeine which was not particularly found in the centre of the tablet as observed in the 30 %w/w HPMC tablet.

The HPMC particles before (Figure 5.12BI) and after dissolution (Figure 5.12BII) were quite similar as they were uniformly distributed. They seem not to align to any axis. The co-localisation between HPMC and caffeine clusters are later discussed in more detail (see Section 5.4.2.4).

The changes in normalised ion intensity are demonstrated in Figure 5.13 (left y-axis). The $C_8H_{11}N_4O_2^+$ ions show a marked decline in the count of caffeine pre and post dissolution on both 15 %w/w and 30 %w/w HPMC tablets. For the case of HPMC, CH_5O^+ ion intensities increased from 2×10^{-3} to almost 4×10^{-3} in the 30 %w/w HPMC tablet. The caffeine release at 15 minutes in the 30 %w/w HPMC tablet was about 10 % while it was almost 20 % in the 15 %w/w.

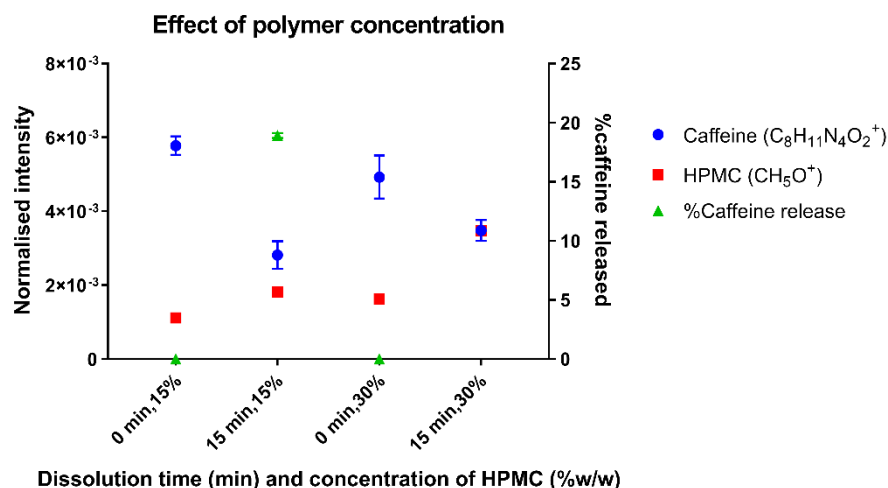


Figure 5.13. ToF-SIMS ion intensity data (left y-axis) highlighting the differences in caffeine ($C_8H_{11}N_4O_2^+$) and HPMC (CH_5O^+) as a function of HPMC concentrations with reported caffeine dissolution (right y-axis).

The caffeine release mechanism of the 30 %w/w HPMC tablet can be explained by combining all the chemical images, ion intensities and the tablet's size changes. Before the dissolution, the distribution of HPMC in the tablet was uniform with no alignment to the y-axis (Figure 5.12BI). With the high content of HPMC, the inter-particle distance between HPMC particles was short and particle entanglement may have occurred. After the tablet was dissolved for 15 minutes, the loss of caffeine intensity (Figure 5.13) and the associated ToF-SIMS ion image (Figure 5.12CII) demonstrates that there were caffeine particles in the outer layer that was already released for the early dissolution. The higher level of HPMC near the tablet's surface may prevent more water ingress. In comparison to the 15 %w/w, the caffeine particles in the 30 %w/w HPMC matrices were kept in the centre acting as a reservoir for extended release.

5.4.2.4 Effect of polymer concentration on formation of HPMC networks using image analysis

The HPMC particles were labelled with different colours to demonstrate the different particle areas of HPMC across the cross-sectioned area. The criteria used for categorising HPMC characteristics in this study are previously shown in 5.3.7.

Figure 5.14 presents the HPMC and caffeine overlay with different displayed colours depending on the HPMC particle area. Before the dissolution, HPMC was uniformly distributed throughout the tablet. As mentioned in Section 5.4.2.3, the HPMC clusters and networks had been observed in the 30 %w/w HPMC matrix since there was no dissolution. A possible explanation for this finding is that the content of HPMC was sufficient to create the HPMC cluster regions. It can be hypothesised that the migration of water into the hydrophilic matrix may cause the swelling of HPMC particles. Swollen HPMC particles may lead to the connection of HPMC particles in the same local region. As observed in Figure 5.14B AII, the HPMC networks were particularly found on the external boundary of the cross-sectioned surface of the 30 %w/w HPMC tablet. It is clear that the caffeine particles were located in the HPMC network area (Figure 5.14BII). The physical changes of HPMC particles may result in the different distributions of caffeine between the 15 %w/w and 30 %w/w HPMC tablets.

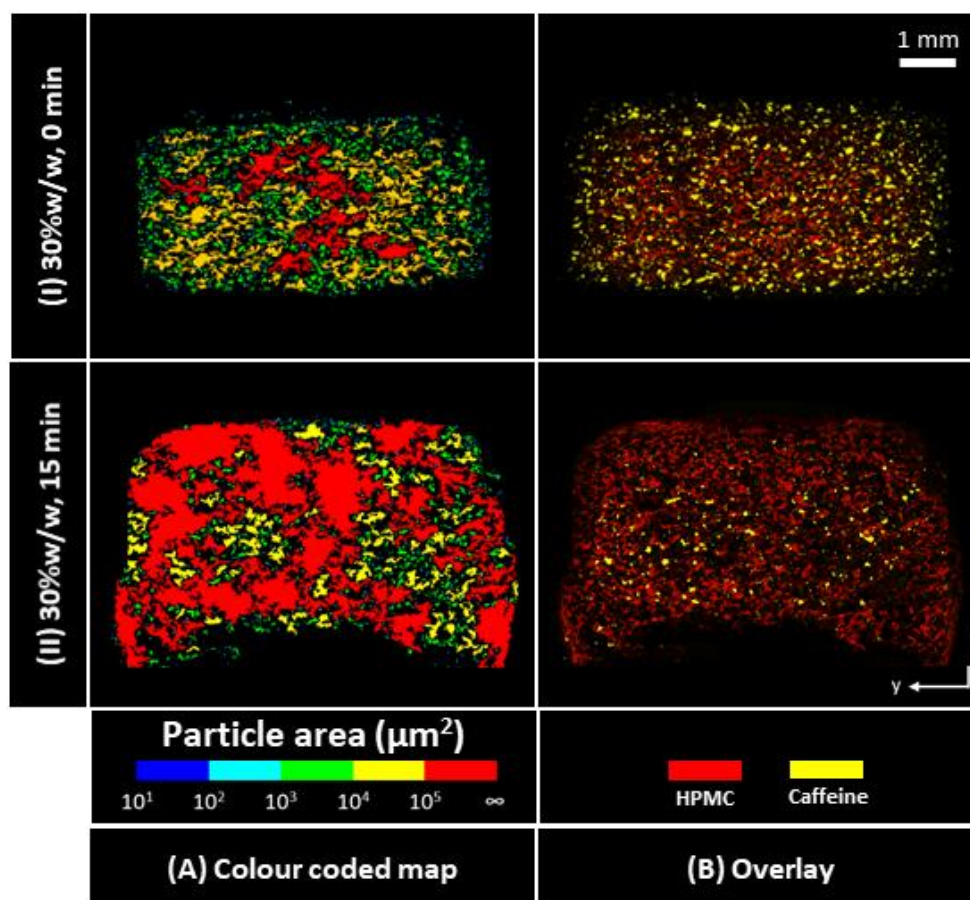


Figure 5.14. (A) Colour-labelled maps of HPMC particles of the 30 %w/w HPMC tablet as a function of dissolution time showing different localisation of HPMC particles depending on HPMC particle area (as displayed as magenta, blue, cyan, green, yellow and red) and (B) HPMC and caffeine overlay.

The count and total area of each group measured using image analysis are presented in Figure 5.15A and B respectively. It can be seen from the results shown in Figure 5.15A that fine and small particles form the majority in all formulations and dissolution time points. The highest total area of the HPMC in 15 %w/w with 15 minutes dissolution was occupied by clusters/connected HPMC particles, followed by HPMC networks and large particles (Figure 5.15B). This result was similar to the 30 %w/w HPMC tablet originally before the dissolution. However, after the 30 %w/w HPMC formulation was dissolved, HPMC networks are observed to dominate (Figure 5.15B). In the 30 %w/w HPMC system, the regions designated as clusters and networks account for 75 % of the total cross-sectioned area.

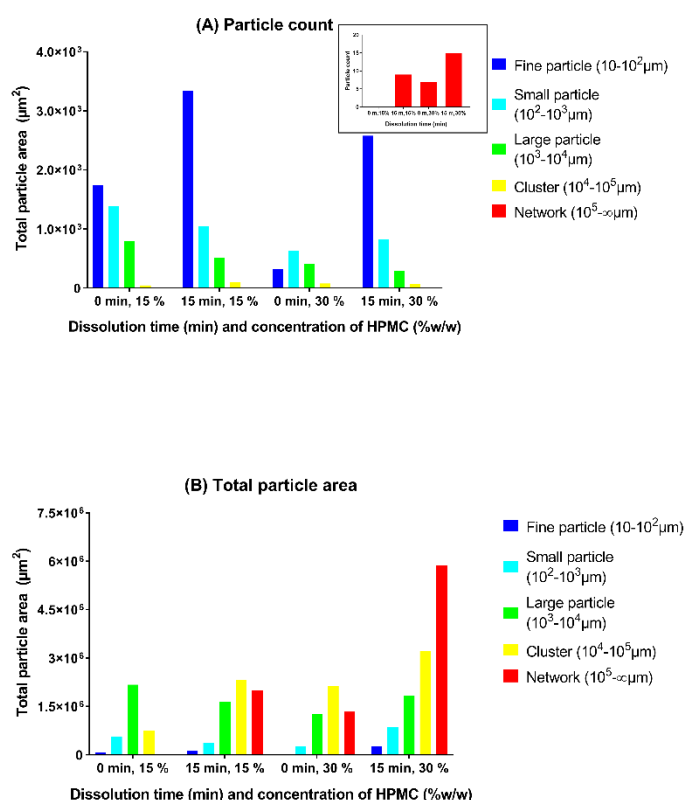


Figure 5.15. Particle count and total particle area of HPMC particles which occupy in each colour including magenta, blue, cyan, green, yellow and red from different HPMC concentrations.

Mass spectrometric imaging was applied with the HPMC matrices for *ex situ* imaging of microstructural changes and component distribution during dissolution. The colour coded map of HPMC tablets shows the locations where the clusters and the networks of HPMC were diagnosed which have never been done in previous research. The present finding also demonstrates that 30 %w/w HPMC is enough to form the complete HPMC gel layers, sustain caffeine particles in the centre of the tablet after dissolution and exhibit extended dissolution time. Interestingly, it seems that the caffeine particles were also located in the areas where HPMC clusters and networks formed.

5.5 Conclusions

The work demonstrates the potential of ToF-SIMS depth profiling and 3D chemical imaging of pharmaceutical tablets. This study has successfully established a suitable approach to depth profiling of the pharmaceutical tablets. It was the first attempt to apply dynamic SIMS to provide the visualisation of HPMC-based matrix tablets with the depth of hundreds of μm . The interconnectivity of HPMC was found in the matrices with 30 %w/w HPMC, demonstrating the existence of percolating polymer clusters. This provides further evidence that 30 %w/w HPMC is needed for controlled release. 3D chemical imaging could avoid the pre-assumptions made regarding 2D imaging as the 2D data is a graphical projection of the 3D object onto the planar surface. However, a common problem that may occur during acquiring data in 3D is the pixel shift. There were two modifications employed to correct the problem in this research. The first was to ensure that the crater region was wide enough so that this could avoid unintended sputtering at the edge of the substrate. The second modification was to adjust the stage height. Thus, the focal position upon the sample of the analysis beam is not changed during data acquisitions.

Additional work has been undertaken which focuses upon analysing observed changes in tablet microstructure following dissolution testing. USP II dissolution apparatus was carried out to perform a release test, the tablets were then dried and subsequently cross-sectioned. ToF-SIMS and image analysis were employed to visualise the chemical distribution of caffeine and excipients in HPMC matrices with respect to dissolution periods and polymer concentrations. It was found that the HPMC gel barrier was not completely formed within 30 minutes dissolution in 15 %w/w HPMC tablets resulting in the burst release of caffeine after 5 min dissolution. This demonstrates that the level of HPMC is not sufficient to control the release of caffeine. When increasing the content of HPMC to 30 %w/w, the complete HPMC layers were developed on the periphery of the tablets and caffeine particles were considerably found in the centre of the tablets. These continuous gel layers could retain the caffeine particles demonstrating a depot for controlled release platforms.

3D chemical imaging and microstructural features of the HPMC tablets after dissolution has provided a useful explanation of the release mechanism of caffeine with regards to the distribution of the hydrophilic polymer. More insights on the chemical distribution as a function of depth would enable measurement of HPMC particle characteristics and distributions in 3D and establish a greater degree of accuracy on dissolution modelling. These applications of ToF-SIMS might help pharmaceutical sectors to understand the release properties of other tablets with different formulation variables.

Chapter 6:

Understanding release performance of proteins and growth factors from enzymatically crosslinked polysaccharide-tyramine hydrogels for cartilage regeneration

6.1 Introduction

From Chapters 3 to 5, a number of analytical techniques were employed to gain chemical information to enhance the understanding of how formulation factors influence the dissolution performance of conventional tablets. In this chapter, the importance of microstructural analysis for understanding the more advanced biological systems, specifically injectable hydrogels, is demonstrated. Using injectable hydrogels as a scaffold is a promising approach for the regeneration of cartilage defects with considerable advantages as described in Section 1.4.2. Therefore, hydrogels have received increased attention as platforms for targeted delivery.

In situ forming hydrogels easily allow protein supplementation and human PL is a cocktail of numerous growth factors and cytokines which could recruit cells and enhance the healing process. It was previously found by Jooybar *et al.* (2019) that PL incorporation in hyaluronic acid-tyramine hydrogel could support cellular activities and induce chondrogenesis of encapsulated mesenchymal stem cells.²⁵⁶ Therefore, PL was chosen to be a medium in the preparation of the gels. Hydrogels with PL can be prepared using enzymatic crosslinking, specifically crosslinked polysaccharide-tyramine conjugates by horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂). Mechanical properties and *in vitro* release of total proteins may be affected by the influence of formulation factors such as type of polymers, concentration of polymers and degree of tyramine substitution.

In particular, the microstructural analysis has never been investigated in the PL-based hydrogel systems. In order to explain the relationship between the formulation factors and the release performance, this study focuses on the ultrastructure and porosity of the formulation to explain the observed release behaviour. TEM and FIB-SEM were employed to investigate the molecular morphology and the structure of the hydrogels. Analysing the hydrogel specimens under cryogenic conditions is important for this study in order to maintain their native state whilst undergoing high vacuum conditions applied during the analysis.

6.2 Aim and objectives

The aim of this chapter is to understand the relationship between gel structure and protein release from *in situ* forming hydrogels. The objectives are:

- (1) Generate new insights into the influence of PL on the mechanical properties of injectable hydrogels and release performance of proteins from the hydrogels.
- (2) Evaluate the effects of formulation parameters on the release of the proteins from injectable hydrogels.
- (3) Determine the micro/nanostructure of hydrogels for illustrating any correlation between the formulation parameters of the hydrogels and release behaviour of proteins from the hydrogels.

6.3 Materials and methods

6.3.1 Materials

Hyaluronic acid tyramine (HA-TA) conjugates with %DS of 16 and dextran tyramine (Dex-TA) with %DS of 10 and 16 were supported by Hy2Care (Enchede, the Netherlands). Platelet lysate (PL) (PLsolution[®]) was purchased from PL Bioscience (Aachen, Germany). Phosphate buffered saline, pH 7.4 (PBS) was purchased from Lonza Netherlands B.V. (Geleen, the Netherlands). Hydrogen peroxide (H₂O₂, 30 %v/v), horseradish peroxidase (HRP, 325 U/mg solid) and sodium azide were obtained from Sigma Aldrich and used as received.

6.3.2 Hydrogel fabrication

Prior to polymer solution preparation, a PL solution was thawed for one hour in a water bath at 37 °C. After thawing, the PL solution was aliquoted and heparin (2 U/ml) was added to prevent coagulation. Subsequently, the PL solution was filtered using a 0.2 µm cellulose acetate syringe filter to remove insoluble particles.

The HA-TA, Dex-TA or HA-TA/Dex-TA (50/50) were dissolved in 100 %v/v of PL or 50 %v/v PL (diluted with PBS) at concentrations of 2.5 or 5 %w/v. In a typical example for the preparation of a 5%w/v HA-TA/Dex-TA (50/50) hydrogel in 50 %v/v PL (1000 µl), a polymer solution of HA-TA/Dex-TA (50/50) with a concentration of 6.25 %w/v was prepared in 800 µl of 50 %v/v PL. 100 µl of a HRP in PBS (40 U/ml) solution was added to the HA-TA/Dex-TA solution. Subsequently, the solution was gently mixed and kept in the fridge overnight (the premixed solution). 100 µl of a freshly prepared H₂O₂ solution in PBS (0.15 %v/v) was then added to the HA-TA/Dex-TA/HRP solution. In the final gel solution, the concentration of HA-TA/Dex-TA, HRP and H₂O₂ was 5 %w/v, 4 U/ml and 0.015 %v/v (a molar ratio between H₂O₂ and TA of 1:4) respectively. After gentle mixing, the final solution was immediately injected into a polydimethylsiloxane (PDMS) mould to produce equally sized multiple gel discs with 8 mm diameter and 1.5 mm height. The design of the PDMS mould

is represented in Figure 6.1. To prevent premature polymerisation the mould was cooled during injection of the hydrogel solution using ice bath. After that, the mould was removed from the ice bath and left at room temperature. Gel discs were used for rheology (Section 6.3.4) and *in vitro* release testing (Section 6.3.6).

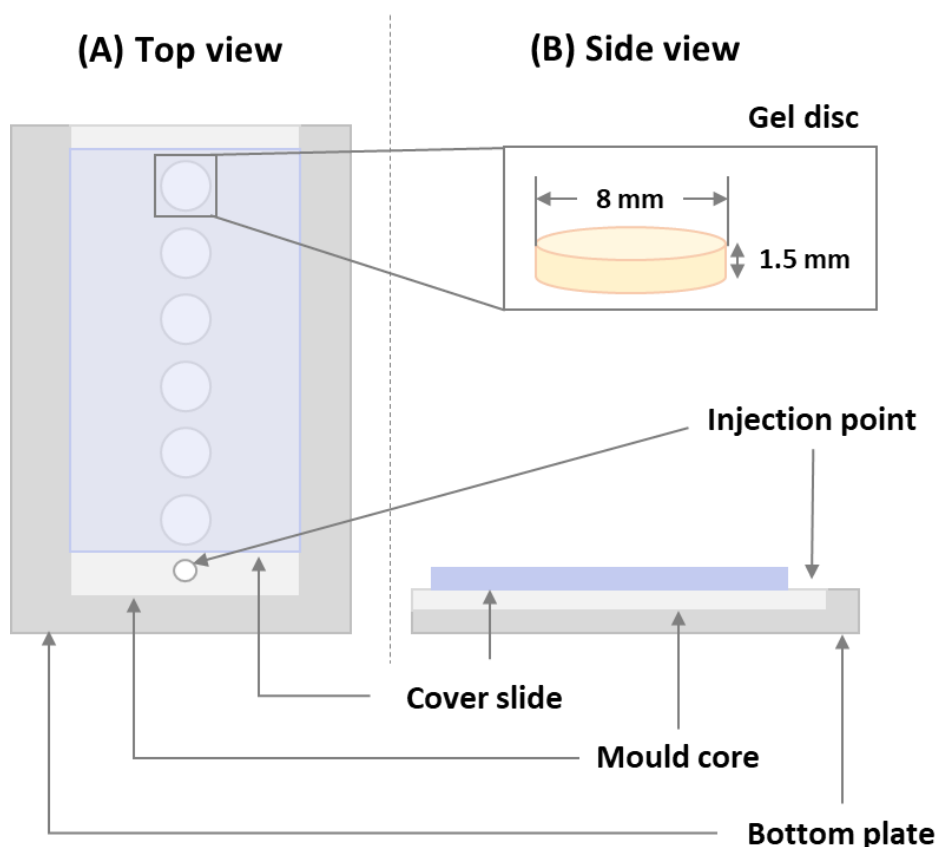


Figure 6.1. Schematic representation of mould design showing the dimension of produced gels with the diameter of 8 mm and height of 1.5 mm: (A) top view and (B) side view of the mould.

6.3.3 Gelation time

The time to form a gel (denoted as gelation time) was determined using the vial tilting method. 225 μl of the premixed solution as described in Section 6.3.2 was later mixed with 25 μl of the H_2O_2 solution. No flow within 1 minute upon inverting the vial was regarded as the gel state.

6.3.4 Rheology

Rheological experiments were carried out with a MCR 301 rheometer (Anton Paar, Graz, Austria) using parallel plates (8 mm diameter, 0 °) configuration at 20 °C in the oscillatory mode. A gel disc was placed at the ground plate and the upper plate was lowered to a measuring gap size of approximately 1.5 mm. The storage (G') and loss (G'') moduli were recorded using a strain of 0.5 % and a frequency of 1 Hz. $\tan \delta$, which is a ratio between G'' and G' , was calculated. The rheological experiments were performed in duplicate.

6.3.5 Total protein content

Coomassie Plus BradfordTM Assay kit (Pierce, Illinois, USA) was utilised to quantify the content of total proteins in platelet lysate. A platelet lysate samples ($n = 2$) were diluted 40 times in PBS. 10 μ l of such solutions and known standard (diluted albumin) solutions with concentrations ranging from 25 to 2000 μ g/ml were pipetted into microplate wells and mixed with 300 μ l of the Coomassie Plus Reagent. Mixing was done for 30 seconds using a plate shaker and the plate was incubated at room temperature for 10 minutes. PBS was used as a blank solution and was prepared as same as sample and standard solutions. The absorbance values of standard and sample solutions measured at 595 nm were collected and deducted by the absorbance value of the blank solution. A standard curve with a second-order polynomial was used to measure the concentration of total proteins in the sample solutions.

6.3.6 *In vitro* release

The hydrogel discs ($n = 3$) were placed in a 48-well plate. A sodium azide solution (0.03 %w/v) in PBS was used as a release medium. Sodium azide was added to prevent microbial growth. The sodium azide solution at specified concentration was claimed for antimicrobial activity.²⁵⁷ 1000 μ l of a freshly prepared dissolution medium was transferred to each well. The solution was prewarmed at 37 °C to simulate body temperature. At specific time points, 15, 30, 45, 60, 120, 180, 240, 360, 480 and 600 minutes (if needed), 10 μ l of the

supernatant in the well was collected to measure UV absorbance. The supernatant was then replaced by the same volume of the medium. The concentration of total proteins was quantified using the same method as shown in total protein content. The release medium was used as a blank solution. The cumulative percentage release of total proteins was normalised to the content of protein theoretically calculated in the samples.

6.3.7 Cryo-TEM

The sample preparation method for a cryo-TEM experiment is described here. The HA-TA or Dex-TA were dissolved in 50 %v/v PL (diluted with PBS) or 100 %v/v PBS at a concentration of 5 %w/v. These polysaccharide-tyramine solutions were mixed with a HRP in PBS (40 U/ml) solution at a ratio of 8:1 overnight. Then, a freshly prepared H₂O₂ solution in PBS (0.15 %v/v) was added to the mixed solutions at a ratio of 1:9 to induce polymerisation of polysaccharide-TA chains. Subsequently, the hydrogels were formed for 1, 3 or 5 minutes prior to freezing. 3 µl of the hydrogels or 100 %v/v PL were injected onto a holey carbon grid. Prior to that, the carbon grid was glow discharged and pre-cleaned. Plunge freezing of the specimens was done to rapidly spread the hydrogels into a thin layer across the carbon support film in liquid ethane, which had been cooled down by liquid nitrogen. Consequently, a vitreous film of gels was achieved to avoid liquid crystalline state of water. Excess solution was removed by blotting with a filter paper for 1.5 s. The samples were transferred to a temperature-controlled container and stored in liquid nitrogen. After the process of sample preparation and plunge freezing, the gel samples were analysed under cryogenic conditions using a JEOL 2100F (JEOL, Japan) at 200 kV equipped with a 914 cryo-tomography holder system (Gatan, Inc, Pleasanton, CA).

6.3.8 Cryo-FIB-SEM

Microscale and nanoscale structural analyses were carried out with a FEI Quanta 200 3D Dual Beam FIB-SEM (Thermo Fisher Scientific, Massachusetts, United States) coupled with a PP2000 Cryo-SEM Preparation System. Prior to analysis,

a polymer/HRP solution which contains 5 %w/v of HA-TA or Dex-TA in PBS/PL (50/50) was premixed overnight with a HRP in PBS (40 U/ml) solution at a ratio of 8:1. Then, this solution was mixed with a freshly prepared H₂O₂ solution in PBS (0.15 %v/v) at a ratio of 9:1. After polymerisation for 5 minutes, the sample was rapidly cooled by coming into contact with a polished metal surface, also known as ‘metal mirror (slam freeze) fixation’. The sample was then transferred under vacuum to the ‘cold stage’ of the ‘preparation chamber’ and sputter-coated with platinum. Milling was done by an ion column which uses a Gallium (Ga) source. The ion beam generated was used for imaging and/or to mill into the specimen while the SEM analyses each fresh layer and/or the milled face. Following that the SEM holder was transferred to the ‘prep stage’ for the sublimation of water by increasing the temperature to -90 °C for about 30 minutes. Finally, the sample was loaded under vacuum into the SEM chamber once again to analyse.

6.3.9 Image analysis

ImageJ 1.52a software (National Institutes of Health, USA) was performed to assess the length of the pores and the porosity of the gels. The type of SEM images was changed to 8-bit and the threshold of the images was adjusted until the entire pores of the gels were highlighted. The porosity of the gels (%) was expressed as a percentage of the open space (void space) area compared to the whole area of the SEM images.

6.4 Results and discussion

Hydrogels were formed *in situ* via the enzymatic reaction between the polysaccharide-tyramine conjugates, HRP and H₂O₂ at room temperature as described in Section 6.3.2. PL was successfully incorporated into the enzymatically crosslinked polysaccharide-tyramine hydrogels in the form of gel media, including 100 %v/v PL and a 50/50 mixture of PBS and PL. Mechanical characterisation tests including gelation time (Section 6.3.3) and rheological properties (Section 6.3.4) were measured to determine how PL influences the physical properties of the hydrogels and to optimise the hydrogels. *In vitro* release performance of the optimised hydrogels was assessed to identify the formulation factors which may impact the release properties of total proteins from the hydrogels (Section 6.4.4). The micro/nanoscale characterisation methods, i.e. electron microscopy, were applied to the hydrogels with different formulation parameters (Section 6.4.5 and 6.4.6). These techniques were employed to investigate the link between formulation parameters of the hydrogels and the release of proteins.

6.4.1 Gelation time

The purpose of this study was to optimise the crosslinking reaction of the tyramine residues in conjugated polysaccharides with the presence of HRP and H₂O₂ with regard to rapid gelation times and the minimal amount of H₂O₂ used. It was found by Rohner *et al.* that the use of high concentrations of H₂O₂ (3 %v/v) may cause an undesirable effect on chondrocytes leading to a reduced cartilage-specific matrix synthesis.²⁵⁸ For hydrogel preparation, it is therefore essential to keep the content of H₂O₂ as low as possible. Gelation times of HA-TA/Dex-TA (50/50) hydrogels at the final concentrations of 2.5 and 5 %w/v which contains HRP (4 U/ml) were measured via the test tube tilting method after addition of a solution of H₂O₂ (0.15 %v/v) as described in Section 6.3.3. It can be anticipated from the use of such diluted concentration of H₂O₂ that this is less likely to have a detrimental effect on cartilage matrix. The molar ratio between H₂O₂ and tyramine residues was 1:4 in the final solution. PBS and PL were used as media for hydrogel preparations. The gelation times for forming

the gels based on PBS and PL media were compared to evaluate the effect of PL addition and the content of polymers on the crosslinking reaction time. The gelation time of each gel with varying concentrations of HA-TA/Dex-TA conjugates and different gel media is shown in Figure 6.2. It can be clearly seen that the delay time for gelation was observed when incorporating PL. This effect of PL supplementation was more sensitive when the lower concentration of polymer conjugates was used. A gel with 2.5 %w/v of HA-TA/Dex-TA (50/50) demonstrated a slow gelation time up to 4-5 minutes when using PL as a medium or a 3.6-fold slower gelation time compared to a PBS-based gel. There was a slight difference of gelation time between PBS and PL at a concentration of 5 %w/v. It can be assumed that PL may contain catalase which is able to competitively decompose H_2O_2 . Regarding the depletion of H_2O_2 , the crosslinking reaction may not be rapid and sufficient to fully react all tyramine groups. This also may lead to gels with reduced mechanical properties.

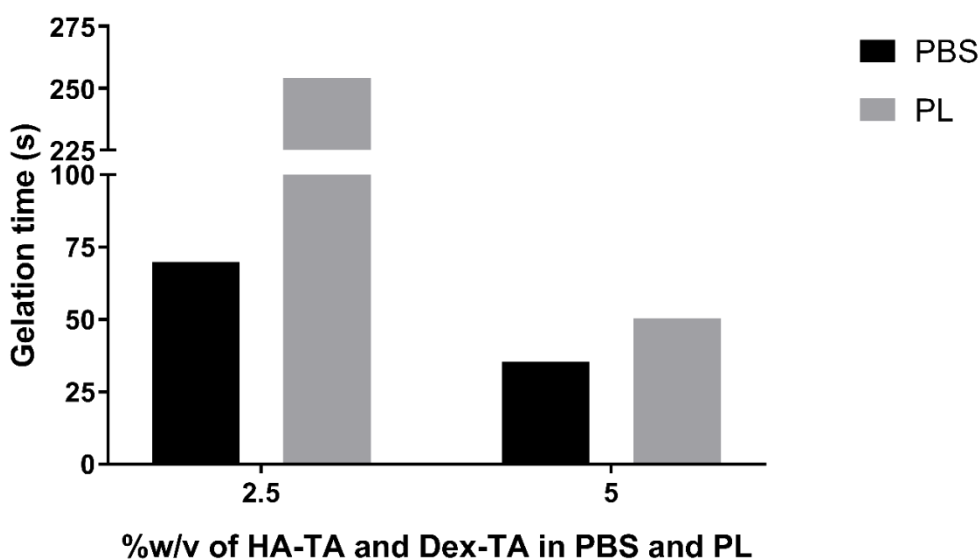


Figure 6.2. Effect of PL and concentration of polymer conjugates on the gelation time of hydrogels composed of HA-TA and Dex-TA (50/50) at 2.5 and 5 %w/v of polymer conjugates in PBS and PL.

To optimise the gelation time and gel properties, PL was mixed with PBS in a ratio of 50:50 to reduce the effect of PL on the gelation time. As H_2O_2 may be consumed by the so-called catalase in PL before being used by HRP in the

crosslinking reaction, the molar ratio between H_2O_2 and tyramine groups was increased from 1:4 to 3:2 to theoretically replace the loss of H_2O_2 due to catalase. In other words, the concentration of H_2O_2 ranged from 0.075 %v/v to 0.45 %v/v for the 2.5 %w/v HA-TA/Dex-TA gels and 0.15 %v/v to 0.45 %v/v for the 5 %w/v HA-TA/Dex-TA gels. This experiment also aimed to evaluate the impact of H_2O_2 on gelation time. As shown in Figure 6.3, the effect of H_2O_2 on gelation time was more pronounced with decreasing polymer conjugate concentration and even more when using 100 %v/v PL as a polymer medium (Figure 6.3A and B). The gelation time was reported almost 260 seconds when the molar ratio between H_2O_2 and tyramine groups was 1:4 and the polymer content was 2.5 %w/v with 100 %v/v PL (Figure 6.3A). With diluted PL medium, i.e. 50 %v/v PL, the gelation time of the gels was constant at approximately 40 to 50 seconds when using 2.5 %w/v of polymer composites (Figure 6.3C). By increasing polymer content to 5 %w/v as shown in Figure 6.3D, the gelation time of the gels with a 50 %v/v PL medium was more rapid than 2.5 %w/v hydrogels. It can be seen that the gelation time of the enzymatic crosslinked gels containing HA-TA and Dex-TA was dependant on the concentration of the polysaccharide conjugate composite, the relative ratios of H_2O_2 to TA groups and the hydrogel media employed.

The constant gelation times which are observed in the diluted PL medium (Figure 6.3C and D) indicates that there might be enough H_2O_2 when reducing PL content for promoting cross-linking between the tyramine residues of the polymers. In summary, the hydrogels prepared from 50 %v/v PL with a polymer composite concentration of 2.5 %w/v or 5 %w/v, demonstrated stable gelation times in less than a minute. Rapid gelation time within a minute would be satisfactory for the hydrogel to remain in an injection site and to be a protein delivery platform for cartilage regeneration. Since fast gelation (less than 60 seconds) was generally observed in the hydrogels comprising 50 %v/v PL (Figure 6.3C and D), a mixture of PL and PBS (50/50) was selected to be a polymer solution medium in subsequent experiments.

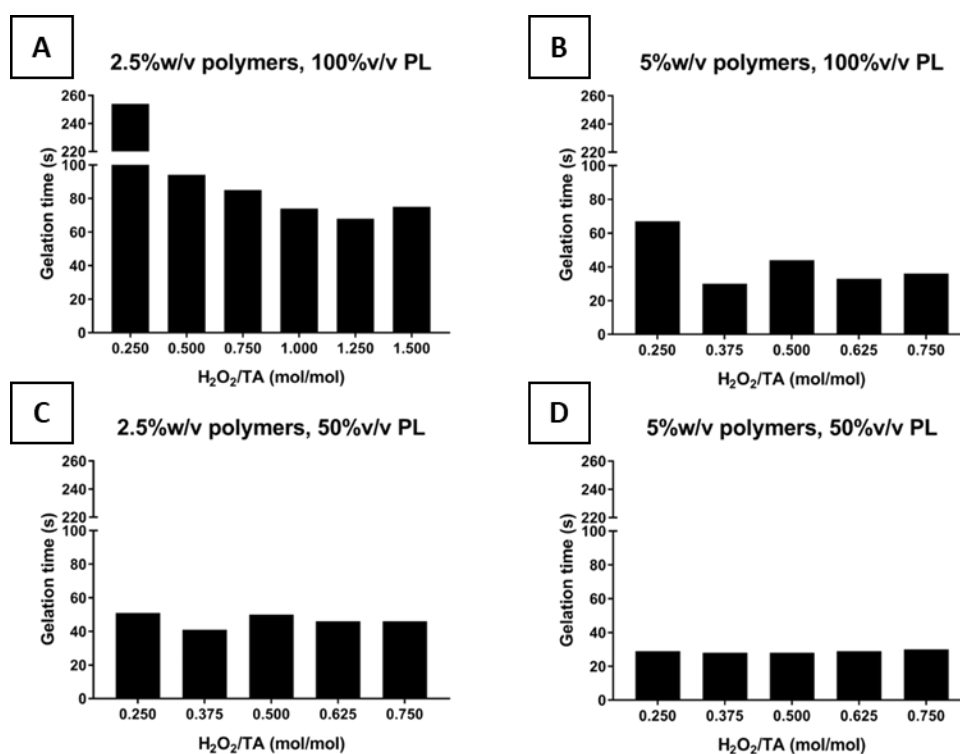


Figure 6.3. Effect of H₂O₂ on the gelation time of hydrogels composed of HA-TA and Dex-TA (50/50) at different content of polymer conjugates and polymer solution medium: (A) 2.5 %w/v of polymer conjugates with 100 %v/v of PL, (B) 5 %w/v of polymer conjugates with 100 %v/v of PL, (C) 2.5 %w/v of polymer conjugates with 50 %v/v of PL in PBS and (D) 5 %w/v of polymer conjugates with 50 %v/v of PL in PBS.

6.4.2 Rheology

Based on the gelation time results (Section 6.4.1), the polymer solutions with 50 %v/v PL in PBS as a medium were used in the preparation of hydrogels and evaluation of their mechanical properties. The rheological results of the 2.5 %w/v and 5 %w/v HA-TA/Dex-TA (50/50) gels can be seen in Figure 6.4A and B respectively. There was a significant effect of a H₂O₂/TA molar ratio on the mechanical properties of the 2.5 %w/v gels. Storage moduli (G'), which represents the elasticity and stiffness of gels, rose approximately 10 times (one log-10 scale) higher when increasing the molar ratio from 0.5 to 1.5. However, at all molar ratios, the 2.5 %w/v gels were observed to be soft and fragile with a gel strength of less than 300 Pa and a loss modulus (G'') of lower than 30 Pa. On the other hand, gels having a strength of 1 to 2 kPa and good handleability were obtained using 5 %w/v polymer composites at any H₂O₂/TA ratio. The damping

factors or $\tan \delta$ defined as G''/G' of the gels were calculated. Their factors were lower than 1 as shown in Figure 6.4A and B and between 2×10^{-2} and 1×10^{-1} indicating that the HA-TA and Dex-TA gels are highly elastic. Compared to the 2.5 %w/v gels, similar $\tan \delta$ values observed in the 5 %w/v formulations indicate that there was similar viscoelasticity even if the molar ratio H_2O_2 between tyramine was increased. These results suggest that the molar ratio between H_2O_2 and tyramine moieties of 0.25 is sufficient in such systems to obtain good mechanical properties. Taking into account the gelation time (Section 6.4.1) and rheology results, the 5 %w/v polymer composites gels prepared at the H_2O_2 /TA molar ratio of 0.25 (0.15 %v/v H_2O_2) were considered to be acceptable in the preparation of gels for the release studies. The lowest concentration of H_2O_2 is preferable as it would also avoid cytotoxicity issues relating to H_2O_2 .

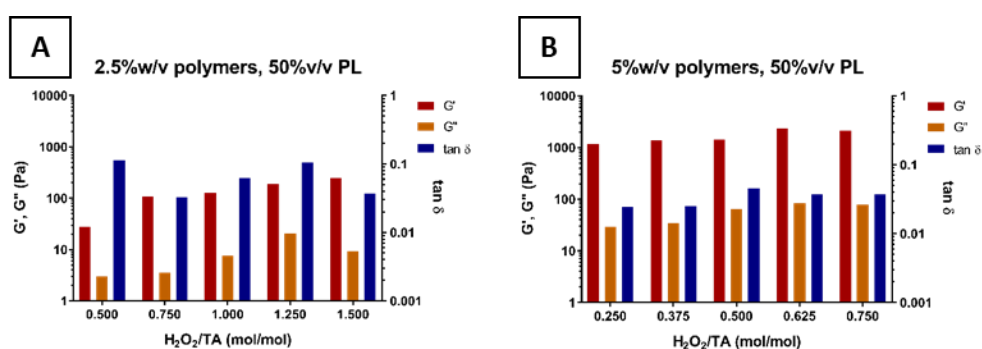


Figure 6.4. Effect of H_2O_2 on the mechanical properties of hydrogels composed of HA-TA and Dex-TA (50/50) in 50 %v/v of PL in PBS as a polymer solution medium using varied polymer concentrations: (A) 2.5 and (B) 5 %w/v of polymer conjugates.

6.4.3 Total protein content

In order to measure the release of total proteins from the injectable gels, the original amount of proteins in PL was evaluated. Coomassie Blue (BradfordTM) protein assay is one of the most widely used methods for protein quantification assay due to easy use, low cost of the reagents, good sensitivity and specificity.²⁵⁹ As mentioned in Section 6.3.5, a series of diluted albumin (BSA) standards were prepared. After blank correction, the calibration curve of UV absorbance values at 595 nm was plotted against the concentration of total proteins (0 to 2000 $\mu\text{g/ml}$). According to a recommendation from a manufacturer

of the Bradford™ assay kit, a second order polynomial was selected as it provides more accurate results than a linear fit. The equation of the calibration curve can be seen in Equation 6.1 with a reported R^2 of 0.995, where x_1 is the concentration of proteins ($\mu\text{g/ml}$) and y_1 is the absorbance value. This curve was used to determine the amount of total proteins in PL samples. The samples were diluted before their absorbance values were measured. It was found that the average concentration of total proteins in platelet lysate samples ($n = 2$) was approximately 32 mg/ml.

Equation 6.1. Calibration curve of protein quantification using Bradford assay.

$$y_1 = -2.57 \times 10^{-7} x_1^2 + 1.01 \times 10^{-3} x_1 - 1.66 \times 10^{-3}$$

In an attempt to simulate release testing conditions, the hydrogel sample was prepared as a round cylinder gel disc with a diameter of 8 mm and a height of 1.5 mm. With the known volume of gel disc (V), the content of proteins in μg per disc (x_2) can be calculated using Equation 6.2, where r is the cylinder's radius (m) and h is the cylinder's height (m), T is the theoretical content of PL (%v/v) in polymer solutions. The theoretical amount of proteins in a single gel disc was approximately 1 mg.

Equation 6.2. The content of proteins in a gel disc.

$$x_2 = \pi r^2 h T x_1$$

6.4.4 *In vitro* release performance and modelling

Previous research investigated the effect of PL on chondrogenic induction (Section 1.4.3).²⁵⁶ The present study focuses on the physical properties of the gels as a result of PL, specifically the release of bioactive proteins. The effect of different formulation variables on *in vitro* release of proteins from the hydrogels was examined. Such variables include the ratio of polysaccharide-tyramine conjugates, their content and degree of tyramine substitution (%DS) in the polymer backbones. Moreover, mathematical modelling was employed to

identify release mechanisms of proteins and determine parameters which may be related to their releases.

6.4.4.1 Effect of ratio of polymers on release of proteins

Due to rapid gelation time and good mechanical properties, 5 %w/v HA-TA, Dex-TA or HA-TA / Dex-TA gels with 50 %v/v PL in PBS were prepared. The H_2O_2 solution with 0.25 mol of H_2O_2 / mol of TA was mixed with the polymer and HRP solutions as this ratio is enough to produce stable gels (Section 6.4.2). Both HA-TA and Dex-TA used in this experiment had a percent tyramine degree of substitution (%DS) of 10. Figure 6.5A shows *in vitro* release profiles of total proteins from gel discs with varied polymer types or ratios. It is clear that the protein release profiles are dependent upon the ratio of polymers used for hydrogel fabrication. The protein release rate was increasingly slower as the HA-TA content was increased. For the 100 %w/w HA-TA gels, the release of proteins was rather linear demonstrating that the release rate was quite constant for the whole period. The protein release was not completed within 8 hours of the test.

To compare the extended release performance among all formulations, the mean time for 80% protein release ($T_{80\%}$) was extrapolated as shown in Table 6.1. It is striking that the HA-TA gels had a $T_{80\%}$ of 493 minutes (8.22 hours) and possessed the highest $T_{80\%}$ among all gels. Figure 6.5A also displays that approximately 90 % of total proteins were released from the HA-TA gel within 10 hours indicating the extended release of proteins. On the other hand, the Dex-TA gels showed a burst release of total proteins at the beginning of the test, especially in the first 60 minutes. Table 6.1 shows the comparison of the burst release performance among hydrogel formulations which includes the protein releases after 15, 30, 45 and 60 minutes (DR 15 min, DR 30 min, DR 45 min and DR 60 min respectively). The release of proteins in the Dex-TA gels was almost 50 % after 60 minutes release test, as observed in Table 6.1 where DR 60 min was 49.55 ± 12.25 %. Until the dissolution time was 4 hours, the release rate of proteins from the Dex-TA gels reached a plateau and finally the proteins were completely released at 8 hours. It can be seen in Figure 6.5A that when the ratio

between HA-TA and Dex-TA of the hydrogels was 50:50, the release performance of proteins in the hydrogels was in between the HA-TA and Dex-TA gels. The release was initially rapid but not as quick as the one found in the Dex-TA gels. This is also evidenced by lower values in DR 30 min, DR 45 min and DR 60 min of the mixed gel compared to the Dex-TA gels, reported in Table 6.1. After a rapid release, the mixed gels exhibited a gradually reduced release rate and their release reached a plateau at 6 hours.

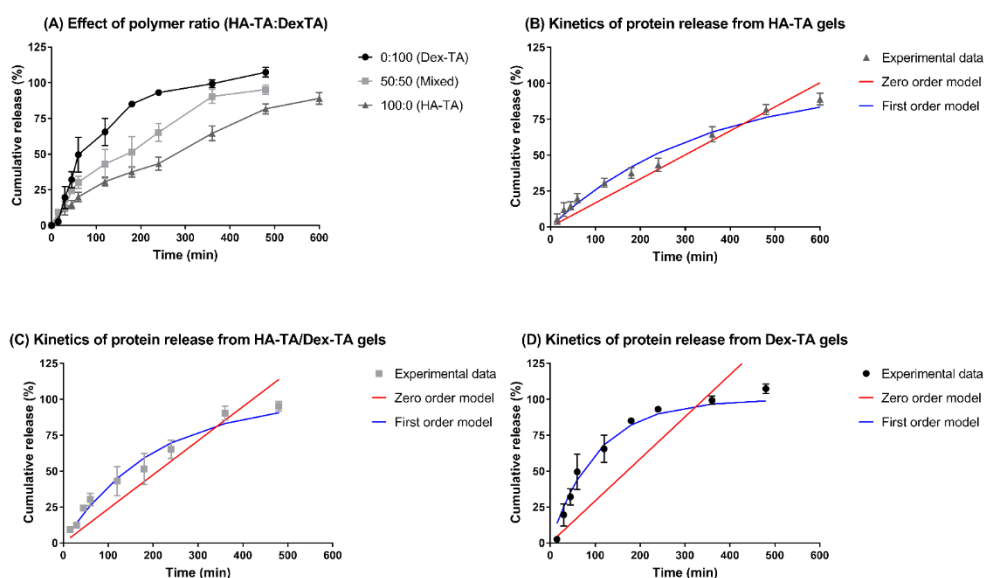


Figure 6.5. Effect of polymer types / ratios on in vitro release of total proteins from hydrogels composed of HA-TA, Dex-TA or HA-TA/Dex-TA (50/50) in 50 %v/v of PL in PBS as a polymer solution medium fitting with zero order and first order kinetics: (A) comparison of the release profiles as a function of polymer types / ratios, (B) kinetics of protein release from HA-TA gels, (C) kinetics of protein release from HA-TA / Dex-TA gels and (D) kinetics of protein release from Dex-TA gels. %DS of Dex-TA is 10.

Table 6.1. Protein release parameters of the hydrogels examined with varied polymer types/ratios for assessment of burst and extended release.

Parameters	Ratio of polymers (HA-TA : Dex-TA)		
	100 : 0 (HA-TA)	50 : 50 (Mixed)	0 : 100 (Dex-TA)
DR 15 min (%)	5.56 ± 3.53	9.50 ± 0.96	2.61 ± 1.25
DR 30 min (%)	12.08 ± 4.80	12.56 ± 2.28	19.60 ± 7.70
DR 45 min (%)	14.66 ± 2.91	24.25 ± 1.61	32.05 ± 5.62
DR 60 min (%)	20.03 ± 3.27	30.29 ± 4.23	49.55 ± 12.25
T _{80%} (min)	493.18 ± 22.94	344.52 ± 13.26	258.99 ± 4.99

DR X min is the drug release (%) after X minutes dissolution and T_{80%} is the time for 80 % drug release.

It is proposed that the hydrogels prepared with different materials may have different crosslinking reactions between tyramine conjugated polysaccharide backbones. This may affect the ultrastructure of each gel. The study of the porosity and micro/nanostructure of hydrogels may explain these findings. More details about the ultrastructure of the gels are described in Section 1.4.4. Another possible cause relates to the charge of hydrogels, where it is generally known that the HA-TA is negatively charged. As a consequence, the electrostatic interaction between oppositely charged proteins and the HA-TA gels could possibly occur leading to protein immobilisation and delayed release rate. The release in the mixed gels was intermediate between the HA-TA and Dex-TA gels demonstrating that the charge of hydrogels may be less prominent compared to the pure HA-TA gel type. The mixture of these two materials may be tailored to modify the protein release profile.

As illustrated in Figure 6.5B, C and D, the dissolution profiles of HA-TA, HA-TA / Dex-TA and Dex-TA hydrogels were fitted to zero-order and first-order mathematical models. The resulting kinetic parameters including T_{80%}, zero-order release kinetic constant (k_0), first-order release kinetic constant (k_1) and R^2 calculated from each model are presented in Table 6.2 and Table 6.3. It is apparent that R^2 values over 0.95 were found in all formulations using the first-order kinetic model. It demonstrates that fitting of protein release in all gel

formulations may be first-order even if different polymer types were used. First-order release kinetics were clearly evident in the Dex-TA gels with a high correlation coefficient (R^2) of 0.9616. It is likely that the mechanism of protein release in the Dex-TA gels could be mainly via diffusion. The release rate may presumably depend on the concentration of total proteins in PL added into the gels. While a good fit using first-order model was observed in the Dex-TA gels, the zero-order model had low goodness of fit ($R^2 = 0.5$). It is observed that as the ratio of HA-TA increased, the fit improved from R^2 values of 0.5 to over 0.9. This suggests that the release of proteins from the HA-TA hydrogels may be almost independent of the proteins incorporated in the hydrogels.

Table 6.2. Protein release modelling parameters of the hydrogels with varied polymer types/ratios calculated using zero-order equation.

Parameters	Ratio of polymers (HA-TA : Dex-TA)		
	100 : 0 (HA-TA)	50 : 50 (Mixed)	0 : 100 (Dex-TA)
$T_{80\%}$ (min)	479.16 ± 23.76	337.56 ± 11.85	273.51 ± 2.03
k_0 (min^{-1})	0.1672	0.2372	0.2925
R^2	0.9236	0.8218	0.5185

$T_{80\%}$ is the time for 80 % drug release, k_0 rate constant for zero-order equation and R^2 is correlation coefficient calculated based upon the zero-order model.

Table 6.3. Protein release modelling parameters of the hydrogels with varied polymer types/ratios calculated using first-order equation.

Parameters	Ratio of polymers (HA-TA : Dex-TA)		
	100 : 0 (HA-TA)	50 : 50 (Mixed)	0 : 100 (Dex-TA)
$T_{80\%}$ (min)	538.14 ± 58.21	325.57 ± 42.23	168.48 ± 21.76
k_1 (min^{-1})	0.0030	0.0050	0.0097
R^2	0.9717	0.9590	0.9616

$T_{80\%}$ is the time for 80 % drug release, k_1 rate constant for first-order equation and R^2 is correlation coefficient calculated based upon first-order model.

6.4.4.2 Effect of polymer concentrations on release of proteins

As the charge of Dex-TA is neutral and the protein release from its gels is characterised as first-order kinetics in its release profile as described in Section 6.4.4.1, the Dex-TA hydrogels with %DS of 16 were used in preparation for this study. The comparison between protein release profiles of the hydrogels consisting of 5 and 10 %w/v Dex-TA is displayed in Figure 6.6A. At the reduced polymer level, the protein release rate increased dramatically as a function of time until reaching a plateau after 120 minutes. Table 6.4 shows parameters relating to the release of proteins. The mean drug release of the 5 %w/v Dex-TA gels was about 33.60 % and 60.56 % after 30 minutes and 60 minutes respectively while it was 15.53 % and 38.22 % with the 10 %w/v. These demonstrate that the initial release of proteins in the 10 %w/w gels was slower, whereas, there was a burst release in the 5 %w/w gels. $T_{80\%}$ of the 10 %w/v gels, contrarily, was almost double the time of the 5 %w/v gels with a complete protein release after 10 hours. From Figure 6.5 and Figure 6.6, it can also be noticed that at least a twofold content of Dex-TA needed to use compared to HA-TA in order to achieve an extended release profile. The higher concentration of the polymers may result in denser networks and enhanced gel strength, leading to slower diffusion rate of proteins to the medium.

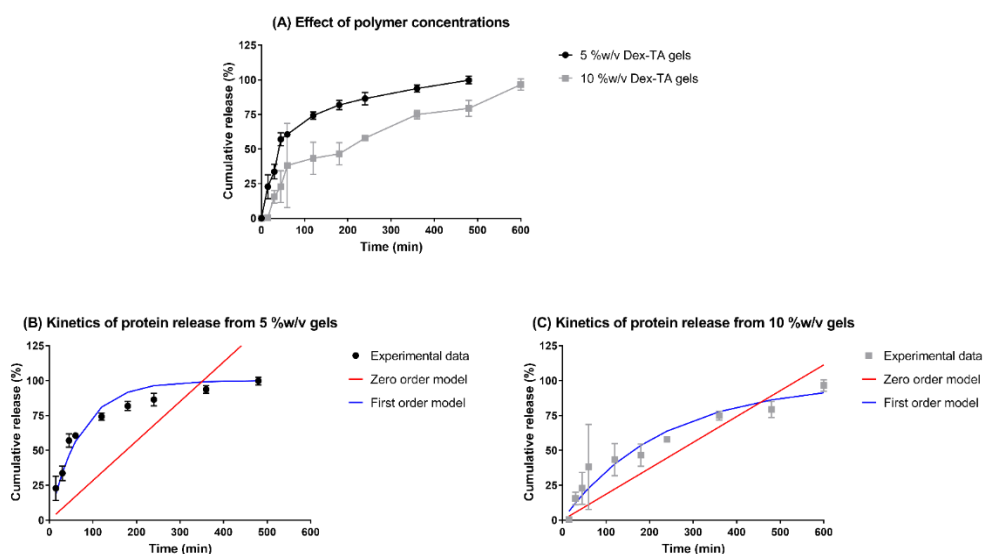


Figure 6.6. Effect of polymer concentrations on in vitro release of total proteins from hydrogels composed of Dex-TA in 50 %v/v of PL in PBS as a polymer solution medium fitting with zero order and first order kinetics: (A) comparison of the release profiles as a function of polymer concentrations, (B) kinetics of protein release from 5 %w/v Dex-TA gels and (C) kinetics of protein release from 10 %w/v Dex-TA gels. %DS of Dex-TA is 16.

Table 6.4. Protein release parameters of the hydrogels with varied polymer concentration of Dex-TA for assessment of burst and extended releases.

Parameters	Concentration of Dex-TA (%w/v)	
	5	10
DR 15 min (%)	22.86 ± 8.67	0.45 ± 0.33
DR 30 min (%)	33.60 ± 5.27	15.53 ± 4.50
DR 45 min (%)	57.05 ± 4.75	22.86 ± 11.50
DR 60 min (%)	60.56 ± 1.54	38.22 ± 30.48
T _{80%} (min)	257.14 ± 14.25	443.21 ± 34.48

DR X min is the drug release (%) after X minutes dissolution and T_{80%} is the time for 80 % drug release.

Modelling the protein release from the 5 %w/v and 10 %w/v Dex-TA data with the zero and first order also was performed and is presented in Figure 6.6B and C respectively. Parameters corresponding to the kinetics such as T_{80%}, R² and kinetic constants are shown in Table 6.5 and Table 6.6. The R² values of less than 0 to 0.6 calculated from the zero-order equation indicate that the release

from the Dex-TA hydrogel systems with the specified concentrations is not independent of the protein concentration in the PL. However, it is speculated that with the higher amount of this polymer, the release of proteins may be more controlled due to the higher density of pores between polysaccharide chains as evidenced by the higher R^2 of 0.6 observed from the 10 %w/v gels. It is clearly observed that the better kinetic model for describing the release mechanism of the hydrogels containing 5 or 10 %w/v Dex-TA was the first-order model with fairly high reported R^2 values of 0.9.

Table 6.5. Protein release modelling parameters of the hydrogels with varied polymer types/ratios calculated using zero order equation.

Parameters	Concentration of Dex-TA (%w/v)	
	5	10
$T_{80\%}$ (min)	282.41 ± 8.10	432.42 ± 30.29
k_0 (min^{-1})	0.2834	0.1856
R^2	-0.6383	0.6452

$T_{80\%}$ is the time for 80 % drug release, k_0 rate constant for zero-order equation and R^2 is correlation coefficient calculated based upon zero-order model.

Table 6.6. Protein release modelling parameters of the hydrogels with varied polymer types/ratios calculated using first-order equation.

Parameters	Concentration of Dex-TA (%w/v)	
	5	10
$T_{80\%}$ (min)	116.38 ± 5.16	389.86 ± 106.20
k_1 (min^{-1})	0.0138	0.0044
R^2	0.8986	0.8706

$T_{80\%}$ is the time for 80 % drug release, k_1 rate constant for first-order equation and R^2 is correlation coefficient calculated based upon first-order model.

6.4.4.3 Effect of degrees of tyramine substitution on release of proteins

The percentage of tyramine residues in the Dex-TA chain may affect the crosslinking efficacy of Dex-TA. However, increasing the degree of tyramine substitution of 6 % had an insignificant effect on the cumulative release of proteins as shown in Figure 6.7. It could be that the increased TA/Dex ratio was too small to impact gel network density, thereby demonstrating a negligible

effect on protein release. Modelling of this data has been discussed as previously shown in Figure 6.5 for the gels with %DS of 10 and Figure 6.6 for the gels with %DS of 16.

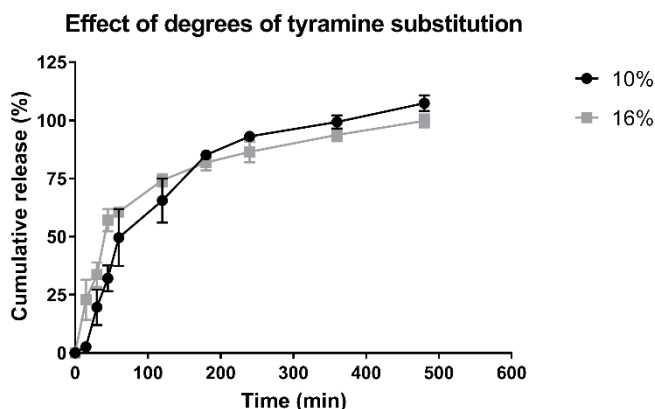


Figure 6.7. Effect of degree of tyramine substitution on *in vitro* release of total proteins from hydrogels composed of Dex-TA in 50 %v/v of PL in PBS as a polymer solution medium.

The most important limitation lies in the fact that Bradford assay used for the quantitative analysis of proteins can only identify and quantify nonspecific total protein content. Further investigation may be done by investigating the specific growth factors relating to the cartilage repair process using other specific protein assays. This would underpin the potential of PL integrated with injectable hydrogels aiming for patients diagnosed with OA.

6.4.5 Molecular morphology study using cryo-TEM

It has been found that the release behaviour of proteins and growth factors is significantly different between the hydrogels prepared with varied gelators, specifically Dex-TA and HA-TA (Section 6.4.4.1). Conventional studies on hydrogel properties such as rheology and gelation time provide information regarding the mechanical properties of the gels. However, the chemical components and the micro/nanostructure of the gels are not considered. Investigation using cryo-EM is of interest to provide a more detailed picture of the molecular organisation.

To prevent water movement and to preserve the native structure of the gels, cryofixation was used in this work. This method offers a lot of benefits for the preparation of hydrogels compared to chemical fixation. For example, cryofixation is rapid (in milliseconds) and there is no need to add a stain or fixative that may interfere with the micro/nanostructure of such hydrogel systems. Cryo-preparation methods for EM is discussed in Section 2.2.5. Cryo-EM techniques applied for the analysis of the hydrogels including cryo-TEM, cryo-SEM and cryo-FIB-SEM were performed to provide insight into the gel structure with various range of resolution.

The morphology of hydrogel samples was investigated employing cryo-TEM. In an attempt to visualise the actual conformation of both polysaccharide-tyramine conjugates, specifically Dex-TA and HA-TA, in forming gels, it is needed to highlight the importance of sample preparation for cryo-TEM analysis. As mentioned in Section 6.3.7, the samples were prepared by rapidly plunge freezing the hydrogel solution into liquid ethane to achieve an ultrathin vitreous film (preferably $< 200\text{ nm}$)²⁶⁰. The gels were directly deposited in the holes of a holey carbon support film. Under cryogenic conditions, the hydrogels were preserved in their native state so that staining or fixing of the specimen is not needed. In the case that the samples are too thick, the beam of electrons cannot pass through and direct observation would be difficult. Polymerisation time in forming gels is a factor which must be considered as with varied polymerisation time, the conformation of the gel may behave differently. Figure 6.8 shows cryo-TEM micrographs of the 5 %w/v Dex-TA hydrogel within PBS:PL (50:50) obtained 1 and 5 minutes after mixing the premixed Dex-TA solution with HRP and H_2O_2 solution. In Figure 6.8A, the presence of small spherical spots can be observed with an average diameter of $34.95 \pm 5.23\text{ nm}$. Some of these spheres can be seen as an aggregate indicated by a yellow arrow. This aggregate is thought to be formed by the isolated spherical spots. These isolated spheres and aggregate are thought to be a dispersed phase (Dex-TA). However, another possibility is that the spherical spots might be protein or protein aggregates originating from the PL.

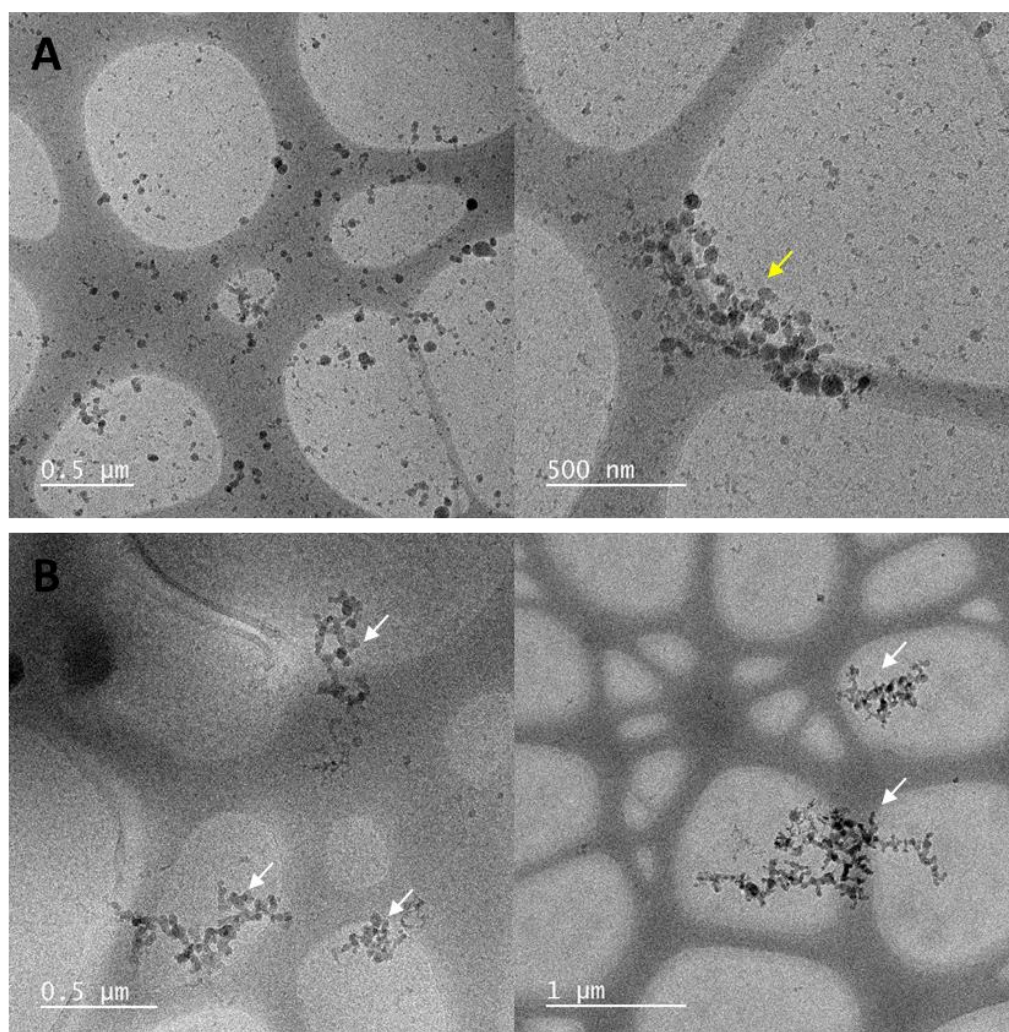


Figure 6.8. Cryo-TEM analysis of 5 %w/v Dex-TA hydrogel in 50 %v/v of PL in PBS as a polymer solution medium with varied polymerisation times: (A) 1 and (B) 5 minutes.

Cryo-TEM was then utilised to analyse PL solution to confirm whether or not proteins could be observed, the result for which is shown in Figure 6.9. None of the spheres found in the TEM imaging of the Dex-TA gel with PL incorporation (Figure 6.8) are observed in the PL solution. This suggests that the dark spheres and aggregates are more likely to be molecules of polymers and their possible polysaccharide chains in the hydrogels. A study led by Kiselev²⁶¹ reported the size and shape of albumin which is an abundant protein found in human platelets. The possible shape of albumin was extended ellipsoids or a non-symmetrical oblate ellipsoid with the size of less than 10 nm. This finding further suggests that the observed spheres in Figure 6.8 may not be albumin in PL and that they

are more likely to be Dex-TA conjugates. However, these spheres could possibly be albumin aggregates.

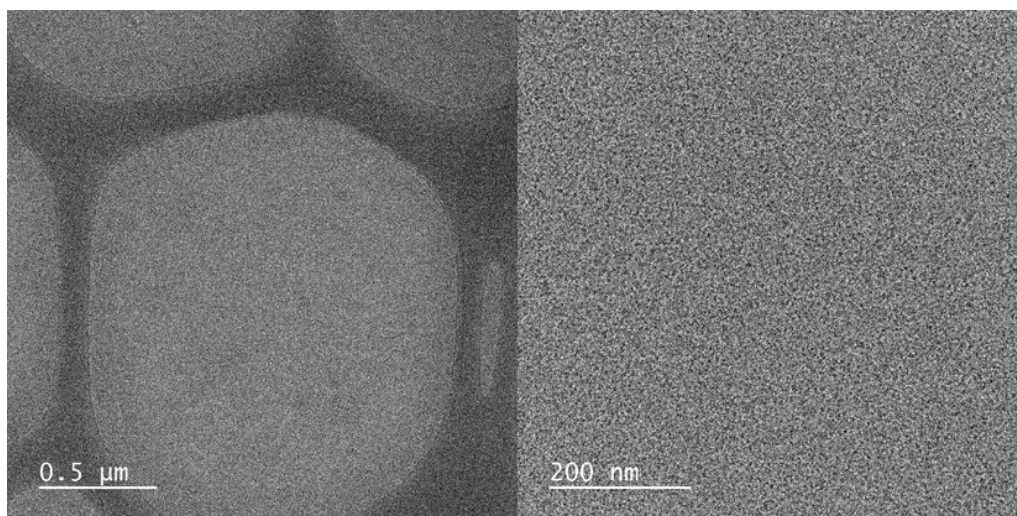


Figure 6.9. Cryo-TEM analysis of PL.

To exclude all the possibilities that protein aggregates in PL could be distinguished using cryo-TEM, the 5 %w/v Dex-TA hydrogel in the absence of PL was examined with cryo-TEM imaging as shown in Figure 6.10. Polymerisation time was varied from 1 to 5 minutes. It can be seen that there were relatively higher numbers of branched polysaccharide chains of the Dex-TA, indicated by white arrows, when using the 50 %v/v PL in PBS as a medium compared to 100 %v/v PL. The hydrogels with higher PL, therefore, requires longer time to polymerise. As mentioned in Section 1.4.2, the formation of branched Dex-TA chains could occur by the HRP-mediated coupling reaction of phenol moieties in TA part of the Dex-TA conjugates.

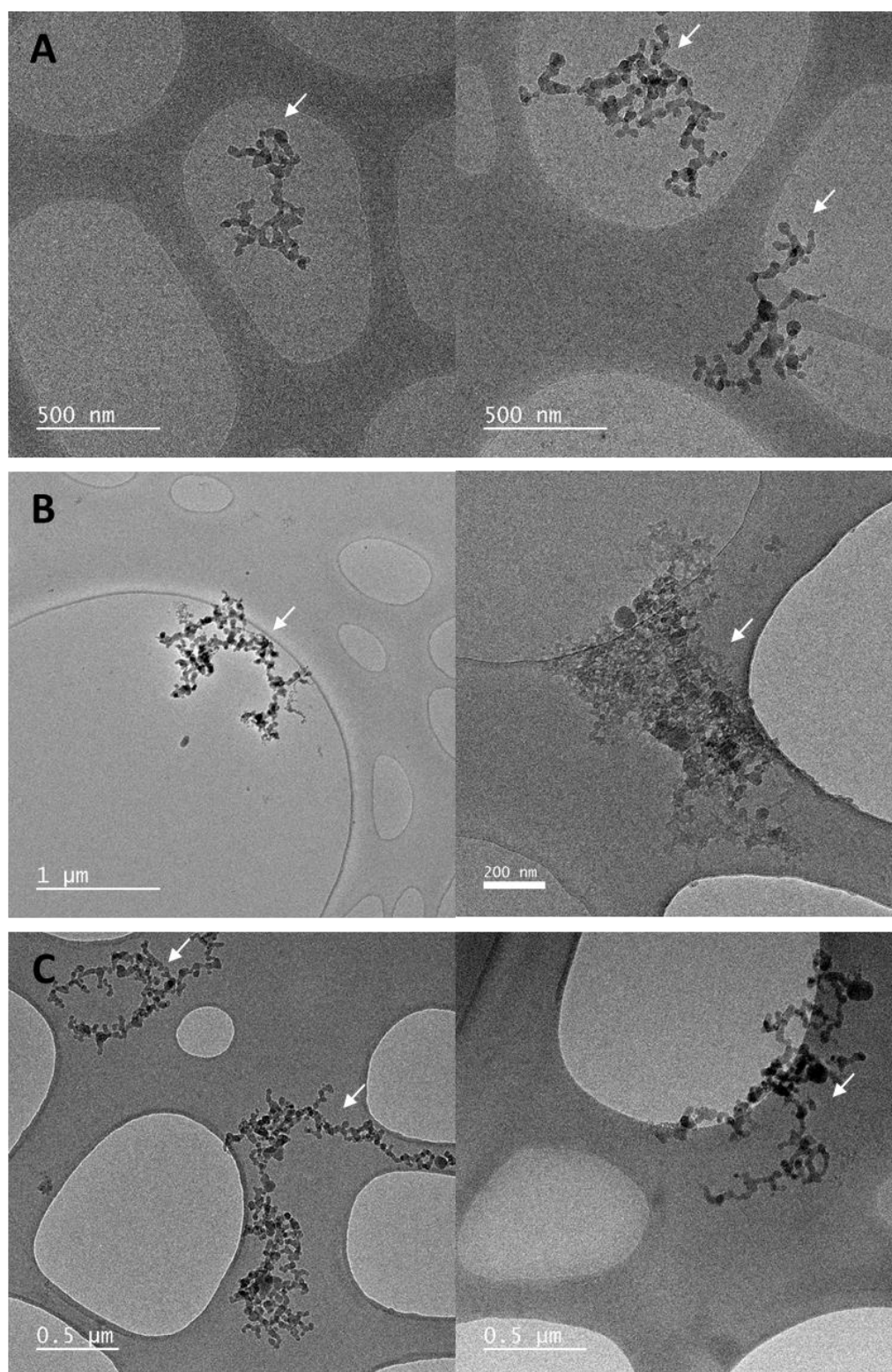


Figure 6.10. Cryo-TEM analysis of 5 %w/v Dex-TA hydrogel in PBS as a polymer solution medium with varied polymerisation times: (A) 1, (B) 3 and (C) 5 minutes.

The molecular structure of the 5 %w/v HA-TA hydrogel with PBS:PL (50:50) as a medium was also determined using cryo-TEM imaging. From the previous

results, the HA-TA/HRP solution was mixed with the H_2O_2 solution and left for 5 minutes before observation. Figure 6.11 shows the HA-TA conjugates distributed across the holes of the TEM carbon grid. The individual spots were aggregated to form branched chains as indicated by white arrows. It can be seen that the Dex-TA chains are more linear compared to the HA-TA conjugates. The conformation of the polysaccharide-TA conjugates were observed using cryo-TEM where the branch structure of the Dex-TA and HA-TA can be found within 5 minutes in the gels containing PL.

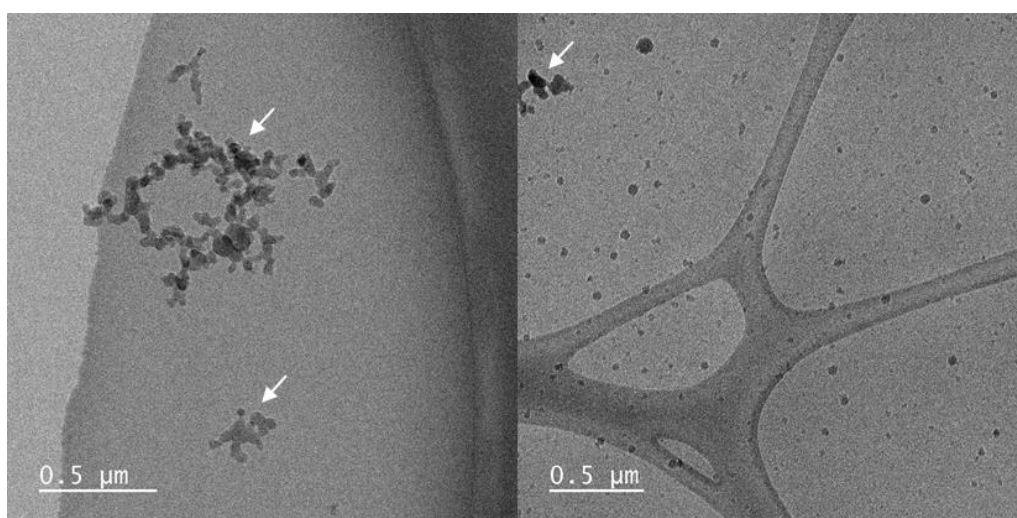


Figure 6.11. Cryo-TEM analysis of 5 %w/v HA-TA hydrogel in 50 %v/v of PL in PBS as a polymer solution medium with 5 minutes polymerisation time.

6.4.6 Microscale and nanoscale characterisation of injectable hydrogels using cryo-FIB-SEM

The degree of porosity may have a substantial effect on the mechanical properties, specifically the release of proteins from hydrogels. This information may only be readily achieved by applying electron microscopy techniques. The porosity of gels was determined using FIB-SEM under cryogenic conditions. The first sample to be analysed was a Dex-TA in PBS and PL (50/50) gel. After completing FIB milling, the sample was subsequently imaged using SEM. It can be seen in Figure 6.12A that the gel is flat with the smooth surface. The sample was then transferred to a ‘prep chamber’ to induce the sublimation of ice, thus revealing the resulting micro/nanostructure of the gel. This process was done by

increasing the temperature from $-175\text{ }^{\circ}\text{C}$ to $-90\text{ }^{\circ}\text{C}$ for 30 minutes in the prep chamber of the FIB-SEM (Section 6.3.8). By applying this novel approach, the ultrastructure of the Dex-TA gels after sublimation was exposed and visualised as shown in Figure 6.12B. The porous features of the Dex-TA hydrogel specimen was apparent in the resulting SEM shown in Figure 6.12B. This structure contains two compartments including the gel strands and pores. These irregular polygonal gel structure exhibited a wide range of pore sizes with an average diagonal of $3.36 \pm 1.07\text{ }\mu\text{m}$. These pores are characterised as macropores with a diagonal line of over 50 nm but less than $300\text{ }\mu\text{m}$. The average porosity of this gel microstructure was $56.68 \pm 1.96\%$, calculated using the method described in Section 6.3.9.

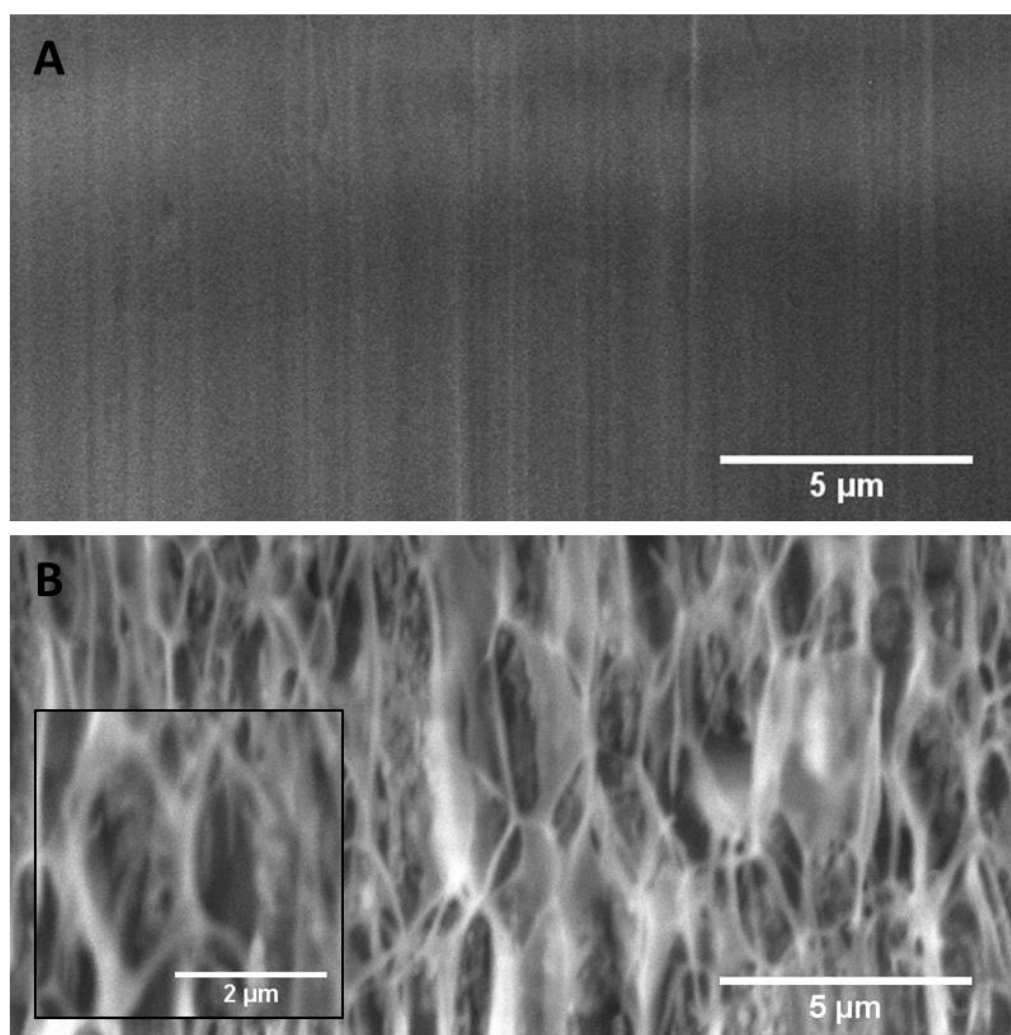


Figure 6.12. Cryo-FIB-SEM of 5 %w/v Dex-TA hydrogels in 50 %v/v of PL in PBS as a polymer solution medium: (A) before sublimation and (B) after sublimation.

The 5 %w/v HA-TA gels with 50 %v/v PL in PBS as a medium were formed *in situ* and were frozen via metal mirror fixation. FIB-SEM imaging of the ultrastructure of these gels was investigated after the process of sublimation. The SEM images obtained for the HA-TA gels before and after sublimation are shown in Figure 6.13A and B respectively. Compared to the Dex-TA gel (Figure 6.12B), the pore characteristics (both shape and size) of the HA-TA hydrogels was significantly different from the Dex-TA hydrogels. Firstly, the pore shapes of the HA-TA appeared to be more commonly elliptical and circular, whereas the Dex-TA showed more irregular polygonal shapes. In term of size, the HA-TA hydrogels showed relatively uniform macropores in the range of 0.62 ± 0.21 μm . It can be seen that the pore size variation of HA-TA hydrogels was also relatively narrower when comparing to the Dex-TA hydrogels. The porosity of the HA-TA hydrogels was 28.00 ± 5.61 % which is half as much as the Dex-TA hydrogels.

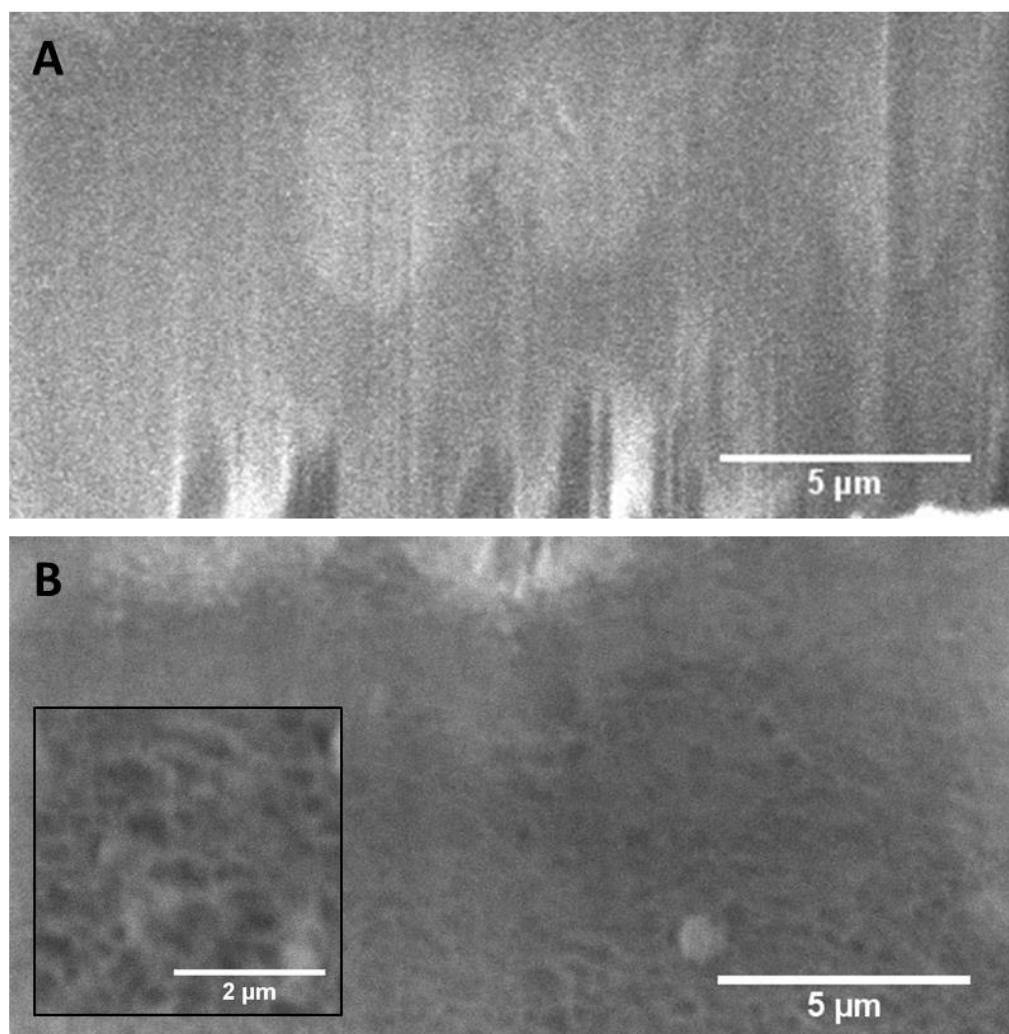


Figure 6.13. Cryo-FIB-SEM of 5 %w/v HA-TA hydrogels in 50 %v/v of PL in PBS as a polymer solution medium: (A) before sublimation and (B) after sublimation.

The larger pore size and higher porosity of the Dex-TA hydrogels may result in the more rapid release of proteins and growth factors from its gel systems as shown in Figure 6.12A. More homogeneous elliptical pore networks found in the HA-TA hydrogels may contribute to the more controlled protein release as it is shown in Figure 6.13A that the total protein release was quite linear. Any large bioactive molecules in PL may be obstructed or delayed by the relatively small pore size of the HA-TA hydrogels. The molecular arrangement of each polysaccharide-tyramine conjugate may have an impact on the interior structure of each hydrogel. As reported in Section 6.4.5, the more extended chains of the Dex-TA were found, compared to the smaller closed rings of the HA-TA conjugates. These may lead to the corresponding gel structure consisting of

different sizes of pores and porosity contents. Using cryo-FIB-SEM and sublimation method, the structure of the hydrogels has been successfully obtained.

6.5 Conclusion

In this study, mechanical properties and *in vitro* release performance of hydrogels containing HA-TA and Dex-TA conjugates were evaluated to understand the effect of PL on the properties of injectable hydrogels. It was found that the addition of PL significantly delayed the gelation time. The percentage of PL in the medium and concentration of the polymer mixture were then optimised. It was found that the gelation time and rheological properties of 5 %w/v of HA-TA/Dex-TA (50/50) hydrogels with 50 %v/v of PL as a medium in a polymer mixture solution were independent with the molar ratio between H_2O_2 and TA. This indicates sufficient H_2O_2 concentration. This formulation was used in preparation of hydrogels for further experiments in order to determine the effect of formulation factors in the release tests.

There are three series of experiments in the release study including the study of polymer ratio, polymer content and degree of tyramine substitution. The HA-TA gels showed the slowest release whereas total proteins were released faster from the Dex-TA gels indicating the requirement for micro/nanostructure analysis. The effect of polymer network density was achieved by changing the polymer level and degree of tyramine substitution in polymer-tyramine conjugates. Extended release was noticeably seen by increasing polymer concentration while an increase in tyramine substitution by 6% only shows a negligible effect.

Molecular morphology and microstructural analysis were conducted to explain the relationship between the polymer types and the release performance of the proteins. It was found using cryo-TEM that the molecular morphology of the HA-TA and Dex-TA was significantly different, whereby the HA-TA gels exhibited more branched chains than the Dex-TA. The molecular morphology may have an impact on how the polysaccharide molecules arrange to form the gels. The ultrastructure analysis was achieved by performing cryo-FIB-SEM and increasing the temperature in the SEM unit to sublimate ice until the pores were exposed. The faster release performance of growth factors and cytokines from the Dex-TA gels can be explained by the higher porosity and larger irregular shaped pores of the Dex-TA gels.

Chapter 7:

Conclusions and future work

7.1 Research summary

The main aim of the work in this thesis was to determine the importance of microstructural analysis in elucidating the release behaviour of active substances from two hydrogel systems, conventional tablets and *in situ* forming hydrogels. For the tablets, HPMC-based matrix tablets with caffeine, as a model API, were prepared and cross-sectioned. ToF-SIMS imaging was found to be the primary technique utilised for demonstrating the distribution of key components in the tablets. The formulation microstructure property relationships of tablets were established with HPMC relating parameters found to be indicative of the release behaviour of caffeine from the HPMC matrices with different formulation factors. The linear regression analysis based on the geometrical data of HPMC in the matrices successfully predicted the dissolution.

ToF-SIMS was also used for evidencing the 3D polymeric network identified in the 30 %w/w HPMC tablet. It showed the effect of hydration time and HPMC content on early gel development. The complete barrier of HPMC was illustrated in the 30 %w/w HPMC tablet suggesting that the gel layer was formed from the outer area of the tablet and the API was maintained in a protected central zone. The hydrogel was fabricated *via* a chemical reaction between polysaccharide-tyramine conjugates and HRP under the presence of H_2O_2 . The ultrastructure of *in situ* gelating hydrogels was assessed to study the effect of polymer types. The larger pore size and higher porosity of a gel may result in the more rapid release of proteins and growth factors. Therefore, the evaluation of microstructure is of paramount importance to obtain additional information and complementary information on the drug release mechanisms from hydrogel-based delivery systems.

7.2 Chemical characterisation of tablet formulations: from raw materials to tablets (Chapter 3)

The work in this chapter successfully demonstrated the feasibility of surface chemical imaging in evaluating the microstructure of the representative tablet systems. The morphology and chemical properties of raw materials and tablet matrices were successfully characterised using a range of analytical techniques including optical microscopy, electron microscopy, vibrational spectroscopy and secondary ion mass spectrometry. The method of tablet cross-sectioning was optimised to gain the internal structure of the tablet and limit cutting defects. Using the method established in Chapter 3 enabled the assessment of the distributional homogeneity of key ingredients in tablets in Chapter 4 and 5.

Key findings discussed in this chapter can be divided into two parts. The first one is the characterisation of raw materials, while the latter part involves the tablet microstructure. It was found that the morphology, specifically the shape and size of five ingredients were quite distinctive. However, HPMC and MCC particles demonstrated the similarity in shape and size. Data from raw materials suggested that optical imaging of tablets was not ideal for differentiating one tablet's ingredient from the others. This points to the importance of chemical imaging in characterisations of raw materials and pharmaceutical tablets. Raw materials were used as references for the identification of key components in the tablet matrices. Using CRM and ToF-SIMS successfully diagnosed all tablet components. It was found that distribution of chemicals in the tablets was homogeneous, reflecting appropriate mixing of solid particulate ingredients in tablet manufacture.

7.3 Relationship between tablet microstructure and dissolution performance of caffeine from hydrophilic matrix tablets (Chapter 4)

The work in this chapter utilised the CRM and static ToF-SIMS method developed in Chapter 3 to successfully elucidate the distribution of principal components in HPMC matrix tablets containing caffeine as an active substance. With the focus upon HPMC particles, the tablets were categorised into two series, including concentration (7.5 to 30 %w/w) and particle size (< 45 to >125 μm) series. With increasing concentration of HPMC, more clusters and networks of HPMC were detected using both CRM and ToF-SIMS. Regarding the similarity of compositional distribution developed from CRM and ToF-SIMS, ToF-SIMS was the technique selected for the analysis of the samples in the particle size series. It was found that the networks of HPMC were inversely correlated to the particle size fraction of HPMC. The parameters relating to HPMC were extracted using ImageJ and taken for the correlation analysis with the dissolution of caffeine at 30 minutes. Correlation coefficient analysis shows that the four possible critical parameters indicative of describing drug dissolution include the inter-particle distance, intersection count, sum of perimeter and mean area. The linear regression model based on the inter-particle distance of HPMC and the number of intersections was able to predict the dissolution performance of caffeine with the adjusted coefficient of determination of 0.9609 and mean absolute error of 2.8. The predicted dissolution profiles of the tablets were compared to the experimental values and it was found that they were similar to the reported similarity factor of more than 50. This study has provided an empirical mathematical model based on chemical imaging parameters of the polymer in addition to other established models in the literature.

7.4 Mechanistic understanding toward polymer network formation in governing drug release from hydrophilic matrix tablets (Chapter 5)

In order to describe the dissolution mechanism, static and dynamic ToF-SIMS were applied to HPMC matrix tablets. The 3D visualisation of the tablet was investigated using ToF-SIMS demonstrating the attachment of the HPMC particles. However, caution in interpretation is recommended due to the fact that increasing the depth of analysis increases the possibility of image shifting. This issue was mitigated by the adjustment of sample height in this study, enabling the possibility in-depth profiling to great depths within tablet samples.

In addition, the caffeine tablets were monitored after being transferred into the dissolution environments. Effects of dissolution time and HPMC content were tested. HPMC did not form a complete gel barrier after 30 minutes dissolution in 15 %w/w HPMC tablets, resulting in burst release of caffeine. The drastic loss of caffeine was observed resulting from the insufficient level of HPMC. Gel layers were completely formed in the 30 %w/w HPMC tablets after 15 minutes dissolution. The caffeine molecules were retained in the core of the tablets providing a depot for controlled drug release. The relationship between HPMC levels and the dissolution rate of caffeine in hydrophilic matrix tablets were uncovered using ToF-SIMS, providing insight into the underlying release mechanism in tablet formulations.

Depth profiling and microstructural analysis can provide the pharmaceutical industries with the chance to examine, in unprecedented detail, formulations of particular interest. This wealth of information would support the formulation design of pharmaceuticals.

7.5 Understanding release performance of proteins and growth factors from enzymatically crosslinked polysaccharide-tyramine hydrogels for cartilage regeneration (Chapter 6)

The work in Chapter 6 provided important insights into new advanced delivery systems, specifically *in situ* forming hydrogels for the application of cartilage regeneration. The hydrogels were optimised and prepared by an enzymatic reaction between the polysaccharide-tyramine conjugates, HRP and H_2O_2 at room temperature. PL, which contains essential proteins and growth factors for cartilage growth, was inserted into the gels as the gel media. Selection of gel formulations was made based on the short gelation time (< 1 minute), good handleability, good gel strength ($G' \sim 1\text{ kPa}$) and lowest use of H_2O_2 . The release rate of proteins was tested using UV spectroscopy. The micro/nanostructure of hydrogels was further obtained using a range of secondary electron microscopy techniques. Based on the established criteria, the gel formulation containing 5 %w/v of HA-TA/Dex-TA hydrogels with 50 %v/v of PL in PBS as a medium was chosen for microstructural studies. The content of proteins in PL was approximately 32 mg/ml. The release of proteins from the hydrogel discs was found to be related to the ratio and concentration of polymers. It was interpreted by fitting dissolution data to zero-order and first-order release kinetics. The release of proteins from the Dex-TA hydrogels was faster than the release from the HA-TA hydrogels. The release rate depended upon the concentration remaining in the matrix, similar to the first-order kinetics. Increasing the HA-TA, the release rate was slower and remained almost constant and independent of the content of proteins in the system. The release kinetics were linked to the nanostructural data from cryo-FIB-SEM. The porosity of the Dex-TA hydrogels was almost double the time of the HA-TA hydrogels, resulting in the more rapid release of proteins and growth factors from its gel systems. The work is the first step towards enhancing an understanding of the microstructure/nanostructure of the gel systems which could describe the release performance from such systems.

7.6 Implications, limitations and future work

The microstructure of hydrogel systems has been proven to assist in our understanding of polymer roles in controlling drug release. Determining the properties of APIs and excipients in the matrices would shorten the overall drug development time for the pharmaceutical industries, especially, the generic companies. The research time for formulations development is crucial as the generic pharmaceutical industries are competitive for being the first manufacturer to successfully develop generic products. The pharmaceutical industries also confront a variety of legal and regulatory requirements to ensure the safety and effectiveness of their products. It is then important to understand the issues related to the performance of products. It was suggested that microstructural analysis could help the pharmaceutical manufacturers achieve the desired product requirements and maintain the reproducibility.

Generally APIs and ingredients contain carbon, hydrogen and oxygen in their chemistry. The normal problem of the analysis of organic materials is that the component in the specimen could generate similar secondary ions in ToF-SIMS analysis. In order to resolve each component and avoid the overlapping of the secondary ion peaks, the need for high mass-resolving power is important. As shown in the results (Section 3.4.4) that traditional ToF-SIMS provides relatively poor mass resolution, then the 3D OrbiSIMS which is a hybrid of ToF-SIMS and an OrbitrapTM mass analyser may be recommended. It would provide higher mass-resolving power due to the efficiency in resolving mass (range of 10^5 – 10^6) of the OrbitrapTM analyzer.

Depth profiling and 3D imaging of the tablets using ToF-SIMS is also challenging. The first issue is that tablets are typically multi-component systems. The sputter rate may not be constant between materials, resulting in distort reconstructed depth profiles and 3D images. Due to the fixed geometry of the ion sources, image shifting becomes increasingly pronounced as the sputter time/depth increases. Furthermore, the image shift may be difficult to avoid with the fixed ion sources. However, the stage height adjustment have been applied to account for the removal of the tablet layers. This improvement allows accurate

3D visualisation of other tablet systems. This method may be used to study other tablet systems such as controlled release multi-layered tablets and coated tablets.

The sample preparation method for monitoring the changes in tablet microstructure after dissolution may be not feasible for other tablet systems. The evaporation of water during drying may affect the microstructure of tablets and deviate the tablet from its native state. This would be improved by freezing the sample and analysing it under cryo-conditions.

The use of elemental and molecular analysis of ToF-SIMS is of particular interest. Other potential future work that ToF-SIMS could be applied in the field of pharmaceutical tablets includes contaminant identification and surface and interface analysis of coating layer in multiple-layered tablets, for example.

The microstructure of the hydrogels has demonstrated evidence to support the release of PL. However, the cryo-FIB-SEM is intrinsically lacking in chemical specificity. Further research might apply other chemical imaging techniques (e.g. cryo-ToF-SIMS and cryo-3D OrbiSIMS) to visualise the distribution of the chemicals in the systems. This approach would probe how the active substance localises after exposure to media.

Chapter 8:

Appendix

8.1 Raman spectra of MCC

It can be observed from the spectra in Figure 8.1 that fluorescence strongly interfered the spectra of Avicel PH102 (MCC) in the 532 nm laser. This incident can occur as the Raman Effect is inherently weak and the MCC molecules might be excited to an electronic excited state by the absorption of the laser light. To lessen the effect of fluorescence interference, the NIR laser was selected in this study. The spectra regarding the NIR laser were less affected by the NIR laser. This may be explained by the fact that the intensity of the NIR laser is lower than the 532 nm laser.

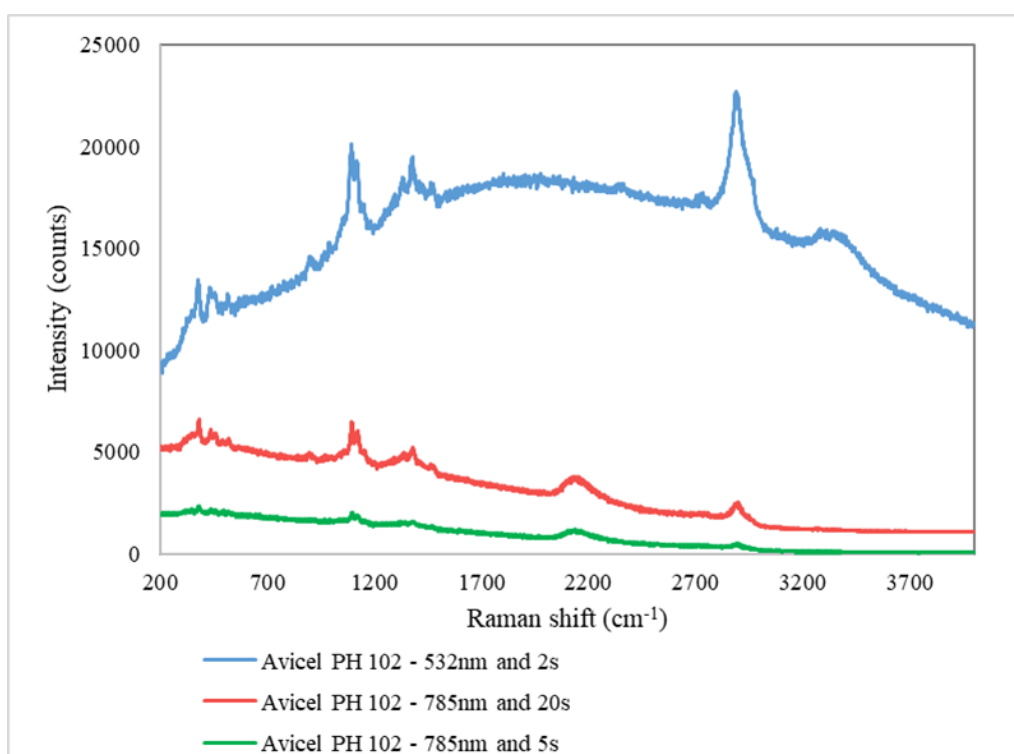


Figure 8.1. Raman spectra showing laser-induced fluorescence observed in MCC.

Chapter 9:

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