Designing nanostructured peptide hydrogels containing graphene oxide and its derivatives for tissue engineering and biomedical applications

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<table>
<thead>
<tr>
<th>Abbreviation</th>
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17
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<td>Rheo-SIPLI</td>
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<td>reactive ion etching</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SAP</td>
<td>self-assembling peptide</td>
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<tr>
<td>SAXS</td>
<td>small angle X-ray scattering</td>
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<tr>
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<td>shear banding</td>
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<td>scanning electron microscopy</td>
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<td>silicon nanowire field effect transistor</td>
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<td>Scanning Probe Microscopy</td>
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<td>SWCNTs</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<td>Trifluoroacetic acid</td>
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<td>valine</td>
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<td>VEVKVEVK</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<td>Y</td>
<td>tyrosine</td>
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<td>overall peptide charge</td>
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<td>zeta-potential</td>
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Progress in biomedicine requires the design of functional biomaterials, in particular, 3-dimensional (3D) scaffolds. Shear thinning, β-sheet based peptide hydrogels have attracted wide interest due to their potential use in tissue engineering and biomedical applications as 3D functional scaffolds. The emergence of carbon nanomaterials has also opened the door for the construction of increasingly functional hybrid hydrogels built from nanofibres and graphene-based materials using non-covalent physical interactions.

The relationship between peptide molecular structure and the formed hydrogel is important for understanding the material response to shear. In particular, the physicochemical properties of peptide based biomaterials will affect the feasibility of injecting them during medical procedures. In the first part of this work, four peptides: FEFKFEFK (F8), FKFEFKFK (FK), KFEFKFEFK (KF8) and KFEFKFEFKK (KF8K) (F – phenylalanine, E – glutamic acid, K – lysine) were designed and used at identical charge to explore the effect of lysine rich β-sheet self-assembling sequences on the shear thinning behaviour and final properties of bulk hydrogels. By varying the peptide sequence design and concentration of the peptide, the tendency of the nanofibres formed to aggregate and the balance of nanofibre junction strength versus fibre cohesive strength could be explored. This allowed the existing theory of the shear thinning behaviour of this class of materials to be extended.

The relationship between molecular structures of nanofibres forming the 3D network and the nano-filler is critical to understand in order to design tuneable and functional materials. In the next part of the work, three rationally designed β-sheet peptides, which form hydrogels: VEVKVEVK (V8), FEFKFEFK (F8) and FEFEFKFE (FE) (V – valine) and five graphene-based materials: graphene oxide (GO), reduced graphene oxide (rGO), three graphene-polymer hybrid flakes: GO with polydiallyldimethylammonium chloride (GO/PDADMAC), rGO with PDADMAC (rGO/PDADMAC) and rGO with polyvinylpyrrolidone (rGO/PVP) were used to form a selection of hybrid hydrogels. Graphene derivatives of the lateral flake sizes of 16.8 ± 10.1 µm were used. Various interactions between the graphene flakes and the peptides were observed that affected the overall mechanical properties of the hydrogels. Electrostatic interactions and π-π stacking, when phenylalanine residues are present, were shown to play
a key role in determining the dispersion of graphene materials in the peptide hydrogels and stiffness of the hybrid materials. In particular, FE with reduced graphene oxide (rGO) and FE with rGO covered with polydiallyldimethylammonium chloride (PDADMAC) thin film formed double network-like hybrid hydrogels due to strong formation of peptide nanofibrillar bridges between adjacent rGO flakes. This corresponded to the 3- and 4-fold increase in the storage modulus (G') of these hydrogels in comparison to controls. FE hydrogels with homogeneous dispersions of graphene oxide (GO) and reduced graphene oxide (rGO) are further shown to be suitable for 3D culture of human mesenchymal stem cells (hMSCs) with no cytotoxicity. These results focus attention on the importance of understanding interactions between the nano-filler and the nanofibrillar network in forming hybrid hydrogels with tuneable mechanical and biological properties, and demonstrates the possibility of using these materials as 3D cell culture scaffolds for biomedical purposes.

Furthermore, graphene oxide (GO) itself is currently used in a number of processes of technological relevance such as wet spinning, injection moulding or inkjet printing to form graphene fibres, composites and printed conductors. Typically, such processes utilise well-aligned layered GO liquid crystal (LC) structures in aqueous dispersions. Flow and confinement encountered during processing affects the alignment and stability of this phase. In the final part of this work, the alignment of GOLCs of two lateral flake sizes (42.1 ± 29.4 µm and 15.5 ± 7.5 µm) were probed under a wide range of rotational shear flow conditions that overlap with the manufacturing processes defined by angular speeds from 0.08 to 8 rad.s⁻¹ (and corresponding maximum shear rates from 0.1 s⁻¹ to 100 s⁻¹), in real-time, using shear induced polarized light imaging and small angle X-ray scattering, both coupled with an in-situ rheometer (Rheo-SIPLI and Rheo-SAXS, respectively). Under certain conditions, a unique pattern in Rheo-SIPLI: a Maltese cross combined with shear banding was observed. This phenomenon is unique to GO flakes of sufficiently large lateral size. The structure formed is attributed to a helical flow arising from a combination of shear flow and Taylor-vortex type flow, which is reinforced by a mathematical model. The orientations prescribed by this model are consistent with anomalous rheopecty observed in Rheo-SIPLI and an anomalous scattering pattern in Rheo-SAXS. With the current trend towards producing ultra-large GO flakes, evidence that the flow behaviour changes from a Couette flow to a Taylor vortex flow was provided, which would lead to undesired, or alternatively, controllable alignment of GO flakes for a variety of applications, including aligned structures for biomedical purposes.
Keywords: Peptide hydrogels, nanofibre network, β-sheet, self-assembly, shear moduli, cells scaffold, graphene materials, stem cells, 3D cell culture, liquid crystal, alignment

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Chapter 1: Introduction

1.1 Motivation for the research

Tissue damage, especially musculoskeletal or cardiovascular injuries can be life-threatening and detrimental to quality of life. In particular, cardiovascular disease (CVD) is a general term that describes a disease of the heart or blood vessels. In this disease, blood flow to the heart is significantly reduced due to a variety of factors, leading to damage of the surrounding tissue and quite often heart attack or stroke.\(^1\) According to World Health Organization (WHO),\(^2\) globally CVD caused 31% of deaths in 2012, being the major contributor of human population deaths worldwide and these diseases continue to do the same currently. Effective repair of tissue damage still remains a challenge.\(^3\)-\(^7\) The progress in biomaterial and tissue engineering fields requires the design of novel functional biomaterials, in particular, functional three dimensional (3D) scaffolds that mimic extracellular matrix (ECM).\(^4\) The materials used must be biocompatible, mechanically tuneable, and offer further attractive physicochemical properties for drug delivery and bio-functionalization. One of the main approaches to obtain appropriate synthetic extracellular matrices (ECMs) is to develop tailorable hydrogels.\(^8\)-\(^14\)

1.2 Hydrogels: 3D biomaterial constructs

Hydrogels are soft-matter constructs in which the swelling agent is water.\(^11, 15, 16\) They are usually formed from amphiphilic polymeric building blocks that assemble to nano-fibrous structures and further interact either physically or chemically to form porous networks. Hydrogels are called physical (sometimes reversible) gels when the networks are held together by molecular entanglements and secondary forces, which include mostly ionic, H-bonding, electrostatic and hydrophobic forces. On the other hand, chemical hydrogels are prepared via crosslinking mechanisms. Hydrogels can be chemically stable or they may degrade and eventually disintegrate and dissolve. The rate of the degradation is highly dependent on the hydrogel building blocks and the external environment.
1.2.1 Types of hydrogels

Hydrogels can be divided into two categories: natural and synthetic hydrogels. Natural hydrogel materials are being investigated for tissue engineering; these materials include agarose, hyaluronan, collagen, fibrin, and synthetic derivatives of natural materials such as chitosan, alginate, silk fibers or peptides. They remain the most physiological hydrogels as they are components of the native ECM. One of the main disadvantages of natural hydrogels is poor control over their final physicochemical properties and difficulty in controlling reproducibility between experiments. The details of the material performance and their dependence on the gelation conditions are often poorly understood. Moreover, due to the natural origin of these materials (bovine fibronogen, rat tail collagen) their composition may vary from one batch to another quite significantly. Also, immune responses due to these materials are challenging, even with natural ECM component materials such as collagens. To overcome this problem, researchers have started to prepare synthetic hydrogels from fully defined building blocks. Whilst these quite often have better performance and reproducibility, the biological responsiveness and compatibility is compromised due to the lack of bioactivity. Since then, scientists have moved on to functionalize synthetic polymers with bioactive moieties (such as RGD or IKVAV peptide) to achieve better biological responsiveness, or formed nanocomposite or hybrid systems of natural hydrogels with synthetic polymers or other organic molecules to obtain better physical characteristics. This leads to infinite possibilities of combining natural and synthetic materials for formation of novel biomaterials.

1.3 Introduction to self-assembling peptide hydrogels

The observations of nature led material scientists to find new ways of forming biomimetic hydrogels. One such observation of alternating ionic hydrophilic and hydrophobic amino acids in proteins in yeast, by Zhang, led to discovery of self-assembling peptides (SAPs). Ever since, he has become a pioneer in the investigations of SAPs and started a new field around forming physical peptide hydrogels.
1.3.1 Peptides and amino acids

Peptides are natural biological molecules, which are short chains of amino acid monomers linked by peptide (amide) bonds. A stable covalent bond is formed between 2 amino acids, when the carboxyl group (COOH) of one amino acid reacts with the amino group (NH$_2$) of another peptide. Peptide length can vary. The shortest peptides are dipeptides, consisting of 2 amino acids joined by a single peptide bond. Any length of peptide with more than 2 amino acids can be regarded as a biopolymer. However, usually sequences where blocks of amino acids repeat in a continuous chain, such as in silk, are referred to as biopolymers. A polypeptide is a continuous chain of less than 50 amino acids. Proteins are large macromolecules, which consist of one or more long chains of amino acid residues. Short and long chains of amino acids under particular circumstances (pH, salt, temperature, light) can form different types of fibrillar structures, which are forms of quaternary structure in proteins due to the self-assembly process, and amyloid in amyloid-related diseases is also fibrillar protein structure. As a minimum, if peptide is uncapped, i.e. if it does not contain any modifications to its end groups, then it has two ionisable groups, the amino group of the N-terminal residue, and the carboxyl group of the C-terminal residue. In addition to these groups there may also be ionisable groups in the side chains of some of the amino acids composing the peptide (only present in lysine, arginine, histidine, aspartic acid and glutamic acid). These charged groups present in amino acids are titratable, therefore the charge on a peptide (or protein) is dependent on pH at which it resides. Simply, the overall charge exhibited by a peptide is the sum of the individual charges present on the amino terminus, the carboxy terminus and any ionisable side chains that are present. In order to calculate the net charge on a peptide, it is important to firstly note the pKa values of the charged residues. When the pH of a solution equals the pKa for an ionisable group, the group exists in 50%:50% mixture of its acidic form and the conjugate base. Also, the further the pH of the solution is from the pKa the higher probability that most of the amino acids present in the structure will be either in acidic or the basic conjugate version. If the pH is less than the pKa, then the acid form of the compound predominates. If the pH is greater than the pKa, then the conjugate base predominates. Then, the theoretical charge state of the peptide can be calculated using the following formula:$^{39}$
\[ |Z| = \sqrt{\left( \sum_i N_i \frac{10^{pK_{a_i}}}{10^{\rho H} + 10^{pK_{a_i}}} \right) - \sum_j N_j \frac{10^{pH}}{10^{\rho H} + 10^{pK_{a_j}}}} \]  

where \( N_{ij} \) are the numbers of groups and \( pK_{a_{ij}} \) the pKa values of the basic (\( i - pK_a > 7 \)) and acidic (\( j - pK_a < 7 \)) groups present on the peptide.

### 1.3.2 Self-assembly

Self-assembly is a common term in use in the modern scientific community to describe the spontaneous, ordered aggregation of particles (atoms, molecules, colloids, micelles, etc.) without the influence of any external forces.\(^{40}\) Large groups of such particles are known to assemble themselves into thermodynamically stable, structurally well-defined arrays. Molecular self-assembly is found widely in biological systems and provides the basis of a wide variety of complex biological structures, hence providing a way of creating biomaterials. Applying the principles of nanotechnology, the function of biological molecules can therefore be manipulated at molecular and macromolecular levels.\(^{41}\)

#### 1.3.2.1 Self-assembly of peptides

Molecular self-assembly is the basis of spontaneous association of peptides built from alternating polar and non-polar amino acids, into well-defined and ordered structures on both nanoscopic (fibres) and macroscopic (networks) levels, as initially shown by Zhang et al.\(^{42,43}\) Research provides many examples showing that on the molecular level, the self-assembly is mediated through weak non-covalent interactions such as van der Waals forces, hydrogen bonding, hydrophobic interactions, aromatic interactions (\( \pi-\pi \) stacking), and electrostatic interactions.\(^{38,44,45}\) These interactions individually are weak, nevertheless, the interplay of all molecular interactions in a given system leads to stabilized conformations of structure, such as a nanofibre. In the last twenty years, researchers have been trying to establish the effect of individual forces on the formation of materials. To this day, the research continues in understanding self-assembly process for forming 3D scaffold materials using natural building blocks, such as peptides, proteins or polysaccharides via bottom-up approach.
1.3.2.2 Short self-assembling peptides as best candidates for forming biomaterials

Interestingly, peptides and proteins are very popular among the biomedical researchers. In particular, short peptides (< 12 amino acids) are very attractive due to the relatively low costs of materials synthesis, associated with the advancements in technology such as solid phase peptide synthesis (SPPS). For example, the cost of 1 g of peptide composed of 8-10 amino acids is similar to 1 g of collagen type 1 (Sigma-Aldrich). Moreover, the library of 20 amino acids allows formation of large number of variations of peptides, leading to formation of new materials. Peptides have simple, relatively short, flexible chains and can be easily manipulated and designed for particular functions, such as bioactivity and physicochemical responsiveness external stimuli, which will be further discussed in the context of formed peptide hydrogels.

1.3.3 Secondary structures

Peptide sequence in the protein and molecular biology sciences is defined as primary structure. It is encoded in the DNA that describes the sequence of amino acids to form a peptide chain. Secondary structure, as the name suggests, constitute the second level of protein structure. This structure is more complex and defines bond angles of the amino acids in proteins. Quite often, these define the functionality and properties of proteins.

1.3.3.1 Alpha-helix and coiled-coils

A common type of secondary structure in proteins is the α-helix (Figure 1), which is a right hand-coiled or spiral conformation in which every backbone N-H group donates a hydrogen bond to the backbone C=O group of the amino acid four residues further along. In proteins essentially all groups capable of forming H-bonds (both main chain and side chain, independently of whether the residues are within a secondary structure or some other type of structure) are usually H-bonded to each-other or to water molecules. Water molecules may also be involved in the stabilization of alpha-helix macrostructures by making hydrogen bonds with the main chain and side chain groups and even linking different peptide groups together. Woolfson et al. have pioneered research in understanding alpha-helices and coiled-coil protein designs. In fact, two or more α-
helices can entwine to form very stable structures, which can have a length of 100 Å or more. These are called coiled-coil structures (Figure 1). Such α-helical coiled-coils are typically found in biological systems, such as myosin and tropomyosin in muscle, fibrin in blood clots or keratin in hair.

**Figure 1**: a) Typical structure of α-helix, where a,b,c,d,e,f, define amino acids of given properties: a,d – hydrophobic, e,g – charged, b,c,f – hydrophilic. Two alpha helices can interact with each other (denoted as α-helix and α*-helix) and form coiled-coil structures as shown in b).

### 1.3.3.2 Peptide Amphiphiles

A typical peptide amphiphile consists of a hydrophilic peptide sequence with attached lipid chains, in this case being a lipopeptide (Figure 2). They were first created by Tirrell et al. in 1995 and since then have been shown to assemble into a variety of nanostructures (Figure 2), including micelles and nanofibres, for biomedical applications by Stupp et al. They showed that peptide amphiphiles are often composed of multiple domains which allow self-assembly into various supramolecular structures. The individual domains associate with each other and this leads to growth of larger assemblies. Initially, Hartgerink and Stupp have designed a peptide amphiphile consists of three distinct areas: a hydrophobic tail, a region of β-sheet forming peptide, and a peptide end moiety designed especially to increase solubility of the whole molecule in water solvent and/or to have a specific bio-functionality. These systems self-assemble into cylindrical micelles by the
combination of hydrogen-bonding between β-sheet forming peptides and hydrophobic tails of the peptides. The formed structures contain high density of the bio-functionalized (and/or water soluble) peptide end at the nanofibre surface.

Later, Hartgerink et al. have developed a new multi domain peptide system, in which the peptide consisted of alternating hydrophobic and hydrophilic amino acids with terminal lysines and this self-assembled into parallel or anti-parallel β-sheets, based on the Zhang’s design. The advantage of this design was that the self-assembly of molecules into nanofibres occurred spontaneously in all solutions of peptide amphiphiles with a very small critical micelle concentration (1 % by weight ≈ 5 mM). They have also shown the dependence of the system on pH and salt presence and used it to change the surface charge of the fibers which triggered gelation. Recently, Hamley summarized the designs and applications of this category of molecules and showed the vast potential of such materials in biomedical and tissue engineering applications.

![Scheme showing typical amphiphilic peptide with a hydrophobic tail and self-assembling peptide sequence](image)

**Figure 2:** Scheme showing typical amphiphilic peptide with a hydrophobic tail and self-assembling peptide sequence (here: C$_{12}$KVIIE, where K: lysine, V: valine, I: isoleucine, E: glutamic acid). Peptide amphiphiles can self-assemble into variety of structures, such as micelles, membrane like aggregates or nanotubes.
1.3.3.3 Short aromatic peptides

Another class of secondary structure formed through self-assembly are short peptides (up to 5 amino acids) that form macromolecular structures via aromatic interactions, mainly π-π stacking. These usually contain phenylalanine (F), tyrosine (Y) or tryptophan amino acids, or fluorenylmethoxycarbonyl (Fmoc) protecting group on the peptide terminal (Figure 3). The stability of aromatic peptides relies on the attractive interactions between π-electrons in aromatic rings, in addition to hydrogen bonds and ionic interactions. Reches and Gazit were the first to observe the self-assembly of short peptides through π-π stacking into ordered structures, such as: nanotubes, hollow spheres and amyloid-like fibres upon dilution from a fluorinated organic solvent. In order to check the true nature of the self-assembly, a variety of diphenylalanine analogues with different C and/or N-terminals were tested in Reches and Gazit peptide design. These ruled out electrostatic interactions between terminal carboxylic acids and amines as the driving force of the self-assembly and indeed confirmed the importance of π-π stacking interactions in the formation of tubular nanostructures structures.

![Selection of aromatic amino acids](image)

**Figure 3**: Selection of aromatic amino acids (Phenylalanine (F), Tyrosine (Y), Tryptophan (W) and fluorenylmethoxycarbonyl protecting group for the peptide terminal (Fmoc). FF denotes a dipeptide made of two phenylalanines, which is a most typical example of short aromatic peptide that forms supramolecular structures such as nanotubes or hollow spheres via π–π stacking.
Since then, Ulijn et al. used the aromatic self-assembly to form hydrogels and responsive biomaterials.\textsuperscript{64, 65} For the past decade, the designs were altered and various analogues of aromatic short peptides were tested. Recently, due to the fact that short peptides consist of only a small number of amino acids, Ulijn et al. developed a computational technique with libraries to screen for useful peptides that can form functional biomaterials.\textsuperscript{47} Such techniques are becoming more often utilized to provide smart design for the novel materials. Nevertheless, this approach at the moment can only be utilised for short peptides due to the exponential increase in number of combinations when adding a single amino acid to a peptide and commensurate increase in the required computational power. Bing Xu et al. have also used the power of aromatic interactions to form a variety of supramolecular materials.\textsuperscript{66, 67} In fact, different secondary structures can be formed from similar peptides. In particular, some secondary structures offer higher stability of the formed hydrogels than others. Bing Xu et al. have further usefully showed the role of aromatic – aromatic interactions in the transition from $\alpha$-helix to $\beta$-sheet secondary structure when mixing two peptides.\textsuperscript{68} In particular, they showed that mixing two peptides with aromatic moieties allows $\pi$-$\pi$ stacking interactions to occur. This in turn, stabilized the overall structure and allows formation of stable $\beta$-sheet structure, in comparison to $\alpha$-helices formed from individual components.

1.3.3.4 $\beta$-sheet and $\beta$-hairpin

Similarly to $\alpha$-helices, hydrogen bonds also stabilize another type of secondary structure in proteins, namely $\beta$-sheets (Figure 4). The hydrogen bonds link together different segments of the protein structure. In other words, hydrogen bonding is not formed between residues close in a peptide sequence (intra-molecular), as in $\alpha$-helices, but by residues close in space, i.e., inter-molecular hydrogen bonding. So disparate regions of a sequence or different peptide chains come together to form a $\beta$-sheet and the individual strands in a sheet are called $\beta$-strands. $\beta$-strands are joined together by at least two or three backbone hydrogen bonds, forming a twisted, pleated sheet (Figure 4). Thus, a $\beta$-sheet consists of several $\beta$-strands, kept together by a network of hydrogen bonds.\textsuperscript{35, 44, 69, 70} $\beta$-sheets can be aligned, such as for instance N- to the C- terminus, and are then named accordingly to the direction of alignment. When all the $\beta$-strands are in the same direction of the $\beta$-sheet, which is from the N- to the C-terminus, they form a parallel $\beta$-sheet (Figure 4). When they point in opposite directions, the $\beta$-sheet is anti-parallel.
Figure 4: Scheme of β-sheet forming peptides in parallel and anti-parallel manner. Dashed lines denote the hydrogen bonding between two peptides. β-sheet forming peptides have tendency to form supramolecular structures such as twisted pleated sheets that can form nanofibres.

Zhang et al. pioneered the research of oligopeptides of alternating hydrophobic and hydrophilic amino acid design which self-assemble to β-sheets. Amphipathic peptides composed of alternating polar and nonpolar residues have a strong tendency to self-assemble into one-dimensional, amyloid-like fibril structures. Fibrils derived from peptides of general (XZXXZ)\(_n\) sequence in which X is hydrophobic and Z is hydrophilic adopt a
recognized β-sheet bilayer, where n defines repeatability of the sequences. The bilayer configuration allows burial of the hydrophobic X side chain groups in the core of the fibril and leaves the polar Z side chains exposed to solvent. This architectural arrangement provides fibrils that maintain high solubility in water and has facilitated the exploitation of self-assembled amphipathic peptide fibrils as functional biomaterials.\(^71\) One β-strand is a fibre created by extended polypeptide chains, typically between 3-10 amino acids long. A method of using de-novo designed peptides made of 8-10 amino acids to create self-assembling β-sheet networks in water was developed (Figure 5).\(^39,72\)-76

![Figure 5](image)

**Figure 5**: Schematic representation of the self-assembling and gelation processes of β-sheet forming peptides. A grey arrow indicates a β-sheet forming peptide sequence. Firstly, β-sheet forming peptides arrange into fibrils. These are stabilized by hydrogen bonding and interact further to form twisted fibres. At sufficient concentration of fibres present (> CGC), fibres interact further via electrostatic interactions, hydrogen bonding, \(\pi - \pi\) stacking and hydrophobic forces to form a self-supported entangled network that holds water, i.e., a hydrogel.

The secondary structure in which there are 2 anti-parallel β-strands, linked by a short 2-4 amino acid sequence, is called a β-hairpin. The loop between the two β-strands is called a β-turn. Turns and loops play an important role in protein 3D structures, connecting β-strands together, or strands to α-helices, or helices to each other. The amino acid sequences in turn regions may be very variable. In some cases, when a loop has some specific function, for example interaction with another protein, the sequence may be conserved. Pochan and Schneider are the leaders in utilizing the β-hairpin design. They have developed their first MAX1 hydrogel (VKVKVKVK^D^PPT^D^VKVKVKVK^D^NH\(^2\)) in 2002,\(^45\) using an intermittent tetra peptide (-V^D^PPT-) designed to adopt type II' turn structure,\(^77\) where P^D^ indicates the D-isomer version of proline. Since then, Pochan and
Schneider have derived a variety of hydrogels and extensively contributed to the biomaterials field. Some of their work is of high importance and will be further discussed in chapter 2 in the context of shear thinning behaviour of peptide hydrogels.

1.4 Peptide hydrogels: development, properties and functions

Peptides that assemble into β-sheets and form fibrous networks can be used to create soft solids, known as hydrogels. As briefly aforementioned, it is relatively easy and cheap to synthesise peptides belonging to this set of commonly used materials. The set of naturally occurring 20 amino acids allows investigation of the fundamental properties of the peptides, such as structure, hydrophobicity, charge state and functionality. This allows the possibility of developing and tuning materials with a wide range of properties.

The self-assembly process to form hydrogels requires spontaneous creation of thick fibre structures and then the entanglement of them into networks. Even though the self-assembly process of peptides is understood at a molecular level, the next part of assembly, that is forming a 3D percolated network, is as yet poorly understood. It is crucial to build a good understanding at all length scales, as the ultimate properties of the created materials depend both on the intrinsic properties of the fibres and the properties of the self-assembled network. A lot of work has been carried out on peptide hydrogels, in particular for use as tissue scaffolds or drug delivery systems, with many using short peptides, based on the alternation of hydrophobic and hydrophilic amino acids (Zhang design), which are well known to spontaneously self-assemble into β-sheet rich fibres that entangle and/or associate above a critical gelation concentration (CGC) to form hydrogels.

30, 35, 38, 39, 42-44, 69, 70, 75, 82-95

In particular, understanding the physical and chemical behaviour of the different residues in these peptides is crucial for selecting the hydrogel properties. Control over the self-assembly process can therefore be used to tune the resulting properties. Moreover, peptide hydrogels can be designed to respond to a range of stimuli, which include: pH, salt concentration, light, temperature or presence of enzymes. The primary structure of peptides is adjustable, which allows control over structure and mechanical properties, as well as allowing the tuning of specific interactions with other molecules or materials. Therefore the ability to control the resulting properties and flexibility in adjusting properties in peptide-based hydrogels makes them ideal candidates for a wide range of applications. Understanding the role of electrostatic
and hydrophobic interactions is of particular interest as the self-assembly process depends on these factors. Interchanging amino acids in the defined peptide sequence and the peptide sequence length itself also affect the final properties of peptide hydrogels. In the next paragraph, these factors will be discussed in the context of self-assembly of β-sheet and hydrogel forming peptides.

1.4.1 Factors affecting self-assembly of β-sheets

A lot of work has been done focusing on the factors affecting β-sheet formation and peptide hydrogel formation. In particular, Zhang, Caplan and Lauffenburger have looked at the variety of factors triggering self-assembly of β-sheet peptides, such as the effect of the charge state of the peptide, electrostatic interactions, pH change and ionic interactions.\textsuperscript{35, 38, 42-44, 69, 70, 78, 82-85, 89, 90, 106-109}

1.4.1.1 Effect of electrostatics, pH and salt concentration

Caplan and Zhang have shown that the self-assembly process of FKFEFKFEFKFE (KFE12) peptide is regulated by the superposition of van der Waals attraction and electrical double-layer repulsion.\textsuperscript{42, 44} This study\textsuperscript{44} not only supported the view that electrostatic specificity over self-assembly conditions is due to like-charge repulsion, but also pointed to the importance of the effect of electrostatics in biological assembly processes. The obtained results were important to other members of the family from which the chosen peptide was derived, and indicated that other oligopeptides with alternating hydrophobic and polar or charged side chains will behave similarly. The further simulation studies of the same group\textsuperscript{42} have shown the effect of pH and charge on the side chains based on the electrostatic double layer theory. The results suggested that side chain interactions are critical in determining the stability and curvature of the formed structure and were the first results indicating such interactions for β-sheet forming peptides. Aggeli \textit{et al.} have also shown that electrostatic interactions play a crucial role in the self-assembly process of the β-sheet peptides.\textsuperscript{38, 85, 89, 90} These studies highlighted the importance of electrostatic interactions, pH change and ionic interactions on the self-assembly process of β-sheet structure. In particular, all of the studies done by Aggeli \textit{et al.} confirmed the importance of electrostatic forces on the self-assembly design of bio-inspired de-novo peptide designs. Additionally, Aggeli \textit{et al.} shows in one of their studies the importance of
the presence of salts and their attenuation of the electrostatic forces. Since then, influence of salts has always been accounted for in the formation of supramolecular structures. Salts indeed have been shown by other groups to have an important effect on the self-assembly process.

1.4.1.2 Effect of hydrophobicity and aromatic interactions

Moreover, the study of the aromatic dipeptide amphiphiles (Fmoc-tyrosine-leucine methyl ester) in aqueous media shows the significance not only of electrostatic effects but also hydrophobicity of the system. This leads to differential hydrophobic interactions which result in differential order and chirality as well as variable mechanical properties of the obtained hydrogels. Other studies, such as Nilsson et al., focused purely on aromatic chain interactions and hydrophobicity. They showed that the role of aromatic π-π interactions in peptide self-assembly is complex and that π-π stacking effects exert a critical influence on the self-assembly of peptides. Nilsson and Bowerman used in their study (XKXK)₂ (where X was a hydrophobic amino acid) peptides as models to reinforce the complex role of aromatic amino acids in promoting peptide self-assembly. In the context of (XKXK)₂ self-assembly, aromatic amino acids did not always more readily induce self-assembly relative to non-aromatic amino acids of similar hydrophobicity, which pointed out the complexity of interactions.

1.4.1.3 Effect of sequence length and replacing amino acids

Bowerman et al. also showed that peptide self-assembly is sensitive to subtle changes in sequence length. They report that truncation of single amino acid from either the N- or C-terminus of the amphipathic (FKFE)₂ peptide results in sequences with different self-assembly behaviour. Truncation of the N-terminal phenylalanine residue gives a peptide, KFEFKFE, which fails to self-assemble in water at acidic pH (pH 3–4). Self-assembly is only observed at neutral pH, which enforces a neutral overall peptide charge that increases peptide hydrophobicity and optimizes cross-strand charge pairing at the hydrophilic face of the β-sheet. Conversely, truncation of the C-terminal glutamic acid residue provides a sequence, FKFEFKF, which readily self-assembles into fibrils with unique broad nanotape morphology relative to the twisted nanoribbons of the parent (FKFE)₂ peptide. Indeed, other groups have reported the effect of length and replacing
amino acids in the peptide sequence on the self-assembly process and final mechanical properties of the hydrogels formed. 

1.4.1.4 Physical properties of hydrogel networks

By replacing amino acids or changing the length of peptide, the intrinsic electrostatic and hydrophobic interactions shift and the resulting mechanical properties differ. Reports on rheological behaviour of the peptide hydrogels usually indicate the formation of nanometer-size network with soft solid like material behaviour and G’ values in the 0.5 kPa to 10 MPa range. For instance, Roberts et al. report on the effects of peptide charge on the self-assembly and gelation behaviour of three octa-peptides: VEVEVKVE (VEK1), VEVKVEVK (VEK2) and VKVKVEVK (VEK3). Their findings confirm the importance of electrostatic interactions and show no differences in the mechanical properties of different peptides when these were prepared at the same concentration and carried the same charge modulus. Studies suggest that replacing one amino acid in the sequence may have an important effect on the overall properties of the formed structure. In particular, Schneider et al. show increased scattering for one of their peptides using spin echo neutron scattering and argue that this is an indication of a heterogeneous network with a tighter mesh size, comparing to other peptides, when a lysine was changed to glutamic acid. The resulting difference in elastic modulus comes from differences in assembly kinetics that came from increased fibrillar branching and physical cross-links rather than a change in the fibril nanostructure or persistence length. Caplan, Zhang et al. describe different length peptides and replacing amino acids in their sequence having an effect on self-assembly. They argue that hydrophobic interactions are of greater effect and are favourable, whereas entropic effects are unfavourable. Hydrophobic interactions are also of greater effect as the peptide length is increased, hence the driving interactions of the self-assembly are shifted when using different sequence of amino acids. Such changes may indeed affect shear moduli by an order of magnitude. This was very recently confirmed by Gao et al. by comparing peptides FEFEFKFK and FEFKFEFK. The swap of the glutamic acid and lysine from positions 4 and 6 resulted in a difference of mechanical properties by an order of magnitude. Indeed, in this study, Saiani et al. show the correlation between changes in amino acids and the network topology formed, which affect the final stiffness of the formed hydrogels. Gao et al.
introduce the new representations of networks, one of uniform fibres and one of heterogeneous (associated) fibres.

1.5 Introduction to graphene-based materials

The use of non-covalent and covalent self-assembly to construct materials has become a prominent strategy in material science offering practical routes for the construction of increasingly functional materials for a variety of applications ranging from electronics to biotechnology. In particular, graphene and its derivatives have shown significant potential as fillers in composite materials as they can give specific functionality and improved properties to the materials. Graphene, also known as graphene layer, single-layer graphene or monolayer graphene, according to the most recent ISO standard is single layer of carbon atoms with each atom bound to three neighbours in a honeycomb structure. It can be best represented as a pure carbon monocrystalline graphitic sheet comprising of single layer of carbon atoms densely packed into a benzene-ring structure (Figure 6). It can be obtained from graphite, which is an allotropic form of the element carbon, consisting of graphene layers stacked parallel to each other in a three dimensional, crystalline, long-range order.

![Figure 6: Structures of graphene (G), graphene oxide (GO) and reduced graphene oxide (rGO). According to the adaptive natural density partitioning (AdNDP) analysis and the electron sharing indices, graphene is aromatic, but its aromaticity is different from the aromaticity in benzene. Aromaticity in graphene is local with two \( \pi \)-electrons located over every hexagon ring.](image)

101, 117 101, 118-120 121 122-124 125
Graphene oxide (GO) is a chemically modified graphene prepared typically by oxidation and exfoliation of graphite bearing oxygen functional groups, such as carboxyl, epoxy or hydroxyl, on their basal planes and edges (Figure 6). Chemical, thermal, microwave, photo-chemical, photo-thermal or microbial/bacterial treatments can be used on GO to reduced oxygen content and lead to production of reduced graphene oxide (rGO).\(^1\) If graphene oxide was fully reduced, then graphene would be the product. However, in practice, some oxygen containing functional groups will remain and not all sp3 bonds will return back to sp2 configuration. Different reducing agents will lead to different carbon to oxygen ratios and different chemical compositions in reduced graphene oxide.\(^1\) As a robust, yet flexible membranes, graphene derivatives provide a large number of possibilities for use in composite materials.

1.5.1 Properties of graphene-based materials

Graphene exhibits unique thermal, electrical and mechanical properties arising from its strictly 2D structure and offers immense potential for technical applications. Graphene has a large theoretical specific surface area (2630 m\(^2\).g\(^{-1}\)),\(^1\) high intrinsic mobility (200 000 cm\(^2\).v\(^{-1}\).s\(^{-1}\)),\(^1\) high Young’s modulus (\(\sim\) 1.0 TPa)\(^1\) and thermal conductivity (\(\sim\) 5000 Wm\(^{-1}\).K\(^{-1}\)),\(^1\) high optical transmittance (\(\sim\) 97.7%)\(^1\) and good electrical conductivity. Graphene-based membranes are impermeable to all gases and liquids (are vacuum-tight).\(^1\) This shows potential for applications in many science fields.

The emergence of carbon nanomaterials and graphene has opened the door for the use of graphene derivatives in a variety of fields that include bio-electronics,\(^1\) tissue engineering,\(^1\) drug delivery,\(^1\) antibacterial materials development,\(^1\) biosensing,\(^1\) gene delivery,\(^1\) and other biomedical applications.\(^1\) Figure 7 summarises these most important properties of graphene based materials that contribute to applications in biomedical fields. The incorporation of graphene based nano-fillers further offers the potential to tailor mechanical strength of native materials, adding binding sites for further bio-functionalization with biological molecules, and supplying additional properties such as conductivity for regulating cell behaviours, such as cell proliferation, differentiation, or protein synthesis, and promotes specific tissue regeneration.\(^1\)
Indeed, Kostarelos and Novoselov emphasized that careful design and proper materials characterization are necessary for selecting carbon nanomaterials to achieve a specific biological outcome. Therefore, understanding the interactions between various graphene-based nano-fillers and the native materials are essential for developing 3D composite scaffolds with desirable characteristics.

1.6 Graphene oxide liquid crystals

1.6.1 Introduction to liquid crystals

Liquid crystals (LCs), as the name suggests are states of matter which are partially characterized by properties of conventional liquids and at the same time some properties of solid crystals. They possess many of the mechanical properties of liquids, e.g., high fluidity. Simultaneously, similar to crystals they exhibit anisotropy in their optical, mechanical, electrical, and/or magnetic properties. The most pronounced feature of LCs is the presence of long-range orientation at order in the arrangement of constituent molecules. The archetypal property of a LC is its anisotropy. Due to the anisotropy in variety of properties, the orientation of the LC molecules can be effectively controlled by weak...
electric, magnetic or shear forces. Consequently, changing the LC molecules orientation, it is possible to change optical and mechanical properties of the medium.

LCs display a large variety of phases, which differ greatly in their structure and physical properties. Mainly, LCs can be divided into thermotropic, lyotropic and metallotropic phases. Thermotropic and lyotropic liquid crystals consist predominantly of organic molecules. Thermotropic LCs undergo a phase transition into the liquid-crystal phase with a change in temperature. On the other hand, lyotropic LCs exhibit phase transitions as a function of both temperature and concentration of the liquid-crystal molecules in a solvent. Finally, metallotropic LCs are composed of both organic and inorganic molecules and their liquid-crystal transition depends on temperature, concentration, and furthermore on the inorganic-organic composition ratio.

Different types of liquid-crystal phases can be distinguished by their different optical properties (such as birefringence). When viewed under a microscope using a polarized light source, different liquid crystal phases will appear to have distinct textures. The contrasting regions in the textures correspond to domains where the liquid-crystal molecules are oriented in different directions. Within a domain, however, the molecules are well-organized.

1.6.2. Graphene oxide liquid crystals: discovery, properties and applications

GO is known to form well aligned layered liquid crystal structures in aqueous dispersions, above certain concentrations and particle sizes. In particular, Sang Ouk Kim has done a lot of research in this area. In 2011, Kim et al. were first to show that the liquid crystallinity of graphene oxide could be maintained upon the decoration of the graphene oxide platelets with nanoparticles or by including an additional polymer component in the solvent medium. They have also shown that the magnetic field or mechanical deformation can induce orientation of GOLCs. Since then, many people have used graphene-based nanomaterials to form well-aligned, self-assembled structures for a variety of applications. Recently, Kim et al. presented a thorough review of GOLCs, starting from a proper explanation of its origin and discovery, going through various phases and arising applications. In LCs, it is known that the geometric shape of the molecules, different supramolecular interactions and nanosegregation of mismatched components are the major driving forces for the formation of various LC phases, such as nematic, smectic, cholesteric, and columnar phases. Indeed, Kim et al. summarised different types of LC phases that have been reported thus far which include: nematic,
lamellar and chiral phases. The main conclusions drawn from the summary was that due to very large shape anisotropy of GO flakes, above some critical concentrations, all graphene based materials, modified or unmodified should form colloidal LC phases. The very first mention of LC phase of pristine graphene was reported only in a strong acidic environment. Since then, the real revolution in the field appeared after discovery of nematic LC phases of GO aqueous dispersions. The main interactions responsible for stabilizing GOLCs is the fact that GO sheets contain large number of hydrophilic groups that allow the flakes to be negatively charged. More about this will be explained in chapter 3 where the comparison of surface properties of five different graphene based materials will be given. Furthermore, after 2011, other research works followed, discovering the mesophases including lamellar and helically chiral LC phases. More importantly, the discovery of these novel phases allowed formation of new materials, such as graphene fibres for high performance applications, prepared by wet spinning. Deep analysis showed that the stability of GOLCs phases is dependent on a few key parameters, which include pH and ionic strength. Therefore, accurate alterations of long range repulsive (or attraction) forces between GO flakes may encourage novel LC phases to appear. This will be further discussed in chapters 4 and 5.

The remarkable potential of graphene material based LCs was already shown fruitfully for a wide range of open, novel applications. Typical examples include, but are not limited to: self-assembled nanocomposites, electroconductive graphene fibres/textiles and high performance supercapacitor electrodes. In order to achieve many of the proposed applications, the macroscopic alignment of GOLCs over large lengths scales must be achieved. Only then, will researchers be able to completely benefit from its anisotropic optical, thermal, electronic and magnetic properties. So far, electric or magnetic field alignment strategies are only verified for very low concentrations of GOLCs. For many of the given applications the processing requires the use of shear. Hence, shear–induced control of alignment could be very advantageous.

1.7 Cytotoxicity of graphene-based materials

Graphene has attracted great interest as a promising nanomaterial for a variety of bio-applications because of its extraordinary properties, especially it’s planar structure and ultra-high surface area (2600 m²·g⁻¹) of graphene facilitate molecular loading and bio-conjugation. Nevertheless, the use graphene and its derivatives in these potential
applications raise safety concerns regarding widespread human exposure. Many reviews over the past few years summarize well recent findings on the toxicological effects and the potential toxicity mechanisms of graphene based materials in bacteria, mammalian cells, and animal models.\textsuperscript{163-171} Graphene, graphene oxide, and reduced graphene oxide elicit toxic effects both \emph{in vitro} and \emph{in vivo}, whereas surface modifications can significantly reduce their toxic interactions with living systems. Recent standardization of terminology\textsuperscript{121}, the graphene roadmap laid down by high academics in the field of graphene\textsuperscript{172} that includes advances in the fabrication methods of graphene-family nanomaterials are showing the effort undertaken to understand and to decrease the adverse effects of graphene-based materials and sets important set of rules for further explorations and translation of this class of materials to medicine.\textsuperscript{173}

\textbf{1.7.1. General introduction to cytotoxicity of graphene-based materials}

There exists the whole selection of graphene derivatives (GDs), which differ in shape, size, surface area, layer number, lateral dimensions, surface chemistry (and charge), stiffness, defect density or quality of the individual graphene sheets, and purity. All of aforementioned properties will significantly influence the interaction of GDs with biological systems.\textsuperscript{163} In general, GDs with small size, sharp edges, and rough surfaces easily internalize into the cell as compared to larger, smooth structures. Typical examples of such materials include carbon nanotubes, which exhibit high cytotoxicity to human cells due to their shape in comparison to GO.\textsuperscript{169} GDs, particularly monolayer graphene, have the theoretical maximum surface area allowing an extremely high capacity for drug delivery. The specific surface area and bending stiffness depend on the number of layers. For biological molecules, the more layers of GDs, the lower the surface area to volume ratio of the material and therefore the lower the adsorptive capacity. The lateral dimensions of GDs, with a range of 10 nm to >100 mm, affect cell uptake mode-of action, renal clearance, blood-brain barrier transport, and many other biological interactions.\textsuperscript{165, 174, 175}

The surface chemistry fluctuates prominently between the affiliates of GDs family, and even before any surface modification it determines the hydrophilicity or hydrophobicity, stability, and dispersibility of these materials in physiological conditions.\textsuperscript{176} GDs can be synthesized by a variety of methods, e.g., mechanical or chemical exfoliation of intercalated bulk graphite,\textsuperscript{177, 178} and therefore, inevitably GDs will contain some impurities such as chemical additives or residual intercalants, including
nitrate, sulfate, and peroxide.\textsuperscript{176} In particular, compared to as-made GO produced by a Hummer’s method,\textsuperscript{179} highly purified GO produces negligible negative effects \textit{in vitro} and \textit{in vivo}, indicating a need for careful cleaning procedures that remove impurities for further biological effects studies and translational medicine.\textsuperscript{180}

1.7.2. Cytotoxicity of GO and rGO

Reports indicate that GDs exert measurable cytotoxicity in both \textit{in vitro} and \textit{in vivo} studies in various types of bacteria, mammalian cells, and animal models.\textsuperscript{163-171} Initial screening of GO- and rGO-based materials for an \textit{in vitro} toxicity assessment commonly uses a variety of cell lines. Data from the literature suggest that exposure to these may result in cytotoxicity and/or genotoxicity in mammalian cells. A detailed analysis of the most recent original research reports along with the earlier review publication unambiguously confirms that graphene in many of its forms and derivatives must be approached as a potentially hazardous material.\textsuperscript{165} It affects a wide range of living organisms, including prokaryotic bacteria and viruses, plants, micro- and macroinvertebrates, eukaryotic mammalian and human cells and whole animals \textit{in vivo}. However, a significant inconsistency and commonly even controversy existing between different experimental findings conducted even in closely related models indicates the demand for further more systematic and coordinated multicentre research investigations including a detailed physicochemical characterisation of the specific graphene materials utilised in each study.\textsuperscript{147, 173}

1.7.2.1 Cytotoxicity of GO

GO is the most comprehensively explored member of GDs family in \textit{in vitro} toxicity studies. Although the first complete study on the toxicity of GO observed neither clear cellular uptake nor noticeable effects on the morphology, viability, mortality, and membrane integrity in adenocarcinomic human alveolar basal epithelial (A549) cells, GO exposure was able to induce oxidative stress at a concentration as low as 10 µg.mL\textsuperscript{-1}.\textsuperscript{181} This was one of the very few reports of a negative cytotoxic response for GO in mammalian cells. Just a few months later, using the same cell line, Hu \textit{et al.} found that GO induced concentration-dependent cytotoxicity to cells, which could be largely reduced by incubation with 10\% fetal bovine serum. This was assigned to high protein adsorption
ability of GO.\textsuperscript{182} Since then, more and more studies focused on the cytotoxicity, genotoxicity and other potential mechanisms of GO action in a variety of human and animal cell lines, including immortalized and normal cell lines, immune cells, stem cells, and blood components. In studies using immortalized cells, the toxicity of GO has been reported in the HepG2 cell line.\textsuperscript{183} Lammel \textit{et al.} evaluated the cytotoxicity of 1-16 µg.mL\textsuperscript{-1} GO using four assays [5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDAAM), alamar blue assay, neutral red uptake assay, and fluorescamine assay]. The authors have found that GO caused a dose-dependent decrease in fluorescence intensity that started at 4 µg.mL\textsuperscript{-1} in the CFDA-AM assay and indicated plasma membrane damage. The loss of plasma membrane structural integrity was associated with a strong physical interaction of GO with the phospholipid bilayer.\textsuperscript{183} Interestingly, TEM and SEM images demonstrated that GO was able to penetrate through the plasma membrane, resulting in altered cell morphology and an augmented number of apoptotic cells. In addition, the authors found the elevated levels of reactive oxygen species (ROS) at GO concentration as low as 1 µg.mL\textsuperscript{-1} and dose-related depletion of the mitochondrial membrane potential which suggested lessened mitochondrial function led to intracellular ROS formation. Among the modes of action assessed, the authors concluded that plasma membrane damage and oxidative stress play crucial roles in GO-induced cytotoxicity.

Yuan \textit{et al.} assessed the cytotoxicity of GO and oxidized SWCNT in HepG2 cells using iTRAQ-2D LC-MS/MS to characterize cellular function.\textsuperscript{184, 185} In their studies, they found that 1 µg.mL\textsuperscript{-1} of both GO and oxidized SWCNTs led to altered protein expression involved in metabolic pathways, redox regulation, cytoskeleton formation, and cell growth, with GO inducing much lower changes in expression in comparison to oxidized SWCNTs.\textsuperscript{184} Furthermore, their compared the cytotoxicity’s of three types of GO: GO-1 and its repeated KMnO\textsubscript{4}-H\textsubscript{2}SO\textsubscript{4} oxidation products, GO-2 and GO-3, in HeLa cells using the MTT assay. The average lateral sizes of GO-1, GO-2, and GO-3 were 205.8 nm, 146.8 nm, and 33.8 nm, respectively.\textsuperscript{186} GO-1 produced significant cytotoxicity at concentrations of 20-100 µg.mL\textsuperscript{-1}, whereas GO-2 and GO-3 exhibited significantly higher viability with higher cellular uptake in HeLa cells, suggesting that the larger sized GO caused greater damage to the cell membrane as compared to the smaller sized GOS.

In another study, the genotoxicity of GO was assessed using the Comet assay. DNA damage, as measured by increased tail length and the percentage of DNA in the tail, were found for all the tested concentrations including 1 µg.mL\textsuperscript{-1}. Notably, 1 µg.mL\textsuperscript{-1} GO caused no visible decrease in cell viability or increase in cellular apoptosis, suggesting that
genotoxicity assays may serve as a more sensitive and representative way to detect the toxicity of GO in mammalian cells. A new finding from this study in comparison to previous studies was that the surface charge of GO and GO derivatives changed their aggregation status as well as their ability to be internalized by cells. It was proposed by the authors that the lower the surface charge of GO, the milder the toxic effect of GO on cells would be.

Majeed et al. show that surface chemistry of GDs plays a major role in their toxicological profile. To demonstrate this, the authors chemically increased the oxidation level of the pristine graphene and compared the corresponding toxicological effects along with those for the GO. X-ray photoelectron spectroscopy (XPS) revealed that pristine graphene had the lowest quantity of surface oxygen, while graphene oxide had the highest, 6.6% and 24% surface oxygen, respectively. These results showed a dose-dependent trend in the cytotoxicity profile, where pristine graphene was the most cytotoxic, whilst the toxicity decreased with increasing oxygen content. Obviously, increased surface oxygen also played a role in nanomaterial dispersion in aqueous solutions or cell culture medium over longer periods. Authors highlight that there is higher possibility that well-dispersed samples might result in graphene entering into cells as individual flakes ~1 nm thick rather than as more cytotoxic aggregates and conclude that changes in graphene's surface chemistry resulted in altered solubility and toxicity, suggesting that a generalized toxicity profile would be rather misleading.

In conclusion, the reviewed data strongly indicates the importance of proper GDs characterization. The toxicity potential of GO can be attributed to its physical forms, surface chemistry, particle size, dosage and the time its exposed to cells. It appears that for many cases the cytotoxicity profiles of GO vary significantly and therefore broad generalizations will rather be difficult to compare to and correlate with existing measurements. Many of these studies focused on flakes of smaller sizes <10 µm of lateral size and with the current trend of producing ultra-large GO, the exact cytotoxicity will still be of great concern and remain unknown until further studies are performed.

1.7.2.2 Cytotoxicity of rGO

The first study of rGO was performed with three cell types: PC12 cells, oligodendroglia cells, and osteoblasts. The authors found that rGO films were more biocompatible than SWCNT. Study performed 2 years later look at the size- and
concentration-dependent cytotoxicity and genotoxicity of rGO and GO nanoplatelets in fresh human mesenchymal stem cells (hMSCs).\textsuperscript{192} Cytotoxicity was measured using three different tests: the fluorescein diacetate cell viability assay, RNA efflux, and the Comet and chromosomal aberration assays. The first test showed significant cytotoxic effects for rGO with an average lateral dimension of 11 nm (the smallest rGO in this study), at the concentrations as low as 1 µg.mL\(^{-1}\) and >1-hour exposure. The largest size rGOs with an average lateral dimension of 3.8 µm exhibited lower cytotoxicity as compared to rGOs with average lateral dimensions of 91 nm and 418 nm, which again, confirmed that the size-dependent cytotoxicity, similarly to previous studies on GO,\textsuperscript{193} RNA efflux assays from cells are indirect indicators of cell membrane damage. These detected size- and concentration-dependent response in rGO-treated hMSCs. The smaller size rGO sheets induced higher RNA effluxes in comparison to the larger size rGO sheets. Furthermore, rGOs generated 13-26-fold higher levels of ROS when compared to the control. It was a direct proof that oxidative stress is one of the key mechanisms involved in rGO cytotoxicity.

In their third study, Akhavan \textit{et al.} showed that rGO flakes with average lateral dimensions of 11 nm and 91 nm caused a significant increase in DNA damage and chromosomal aberration frequency at all concentrations >0.1 µg.mL\(^{-1}\), >1 µg.mL\(^{-1}\), respectively with 1 hour exposure. It is interesting that these concentrations were 10 times lower than the threshold concentration observed in the cell viability test and indicates that rGO may influence cell behaviour on DNA level. The large size rGO sheets at the highest concentration of 100 µg.mL\(^{-1}\) and after a long time exposure of 24 hours induced only slight DNA fragmentation.\textsuperscript{191} This was most likely caused by smaller flakes present in this distribution of size and suggests that the interaction of rGOs with hMSCs strongly depends on their lateral size. It is hypothesize that these results will follow in other types of cells, with the most likely mechanisms of rGO cytotoxicity being oxidative stress and direct contact of the sharp edges with the cells. The sharp edge contact may consequently induce genotoxicity in cells through interaction of the penetrated nano sizes sheets with the nucleus of the cells.\textsuperscript{191}

When comparing to GO, rGO induced significantly higher cytotoxicity in A549 cells.\textsuperscript{182, 194} Hu \textit{et al.} found that rGO nanosheets with a lateral size of 4.6 µm reduced cell viability to 47% and 15% at concentrations of 20 µg.mL\(^{-1}\) and 85 µg.mL\(^{-1}\), respectively.\textsuperscript{194} With a focus of producing antibacterial material, Gurunathan \textit{et al.} synthesized rGO using green methods. Like the others, this rGO prompted higher levels of cytotoxicity,
production of ROS, and loss of membrane integrity in MCF-7 cells as compared to GO. Incubation of MCF-7 cells with both rGO or GO at doses above 60 µg.mL\(^{-1}\) produced striking cytotoxic effects, including significantly decreased cell viability and increased ROS generation.

1.8 Graphene with peptides and proteins: from properties to applications

One method for functionalizing materials without fundamentally changing their inherent structure is using bio-recognition moieties. In particular, oligopeptides are molecules containing a broad chemical diversity that can be achieved within a relatively compact size. Phage display is a dominant method for identifying peptides that possess enhanced selectivity toward a particular target. It is a technique that uses recombinant DNA technology to create bacteriophages with a desired peptide embedded on the surface of their protein shells. Agonists and antagonists of the target peptide can then be identified experimentally, enabling the engineering of antibodies and development of new drugs. Phage display therefore allows selection of peptides and antibodies towards a target using various screening methods. Chemical functionalization of graphene via comprehensive screening phage displayed peptides was recently developed. The obtained results showed that graphene can be selectively recognized even in nanometer-defined strips. Furthermore, modifications of graphene with bio-functional peptides reveal both the ability to impart selective recognition of gold nanoparticles and the development of a sensitive graphene-based trinitrotoluene sensor. Peptides identified from combinatorial peptide libraries have been shown to bind to a variety of abiotic surfaces. Biotic-abiotic interactions can be exploited to create hybrid materials with interesting electronic, biological, or catalytic properties. It was also shown that peptides identified from a combinatorial phage display peptide library assemble preferentially to the edge or planar surface of graphene and can affect the electronic properties of graphene. These mechanisms are extremely useful for identifying the interactions between graphene and bioactive molecules and the surface interaction will be discussed further in this chapter.

1.9 Carbon nanomaterials in tissue engineering

Electrical properties of graphene and carbon nanomaterials are very attractive for scientists to exploit. The two most recent studies that underpin creation of electrically
Conductive materials have been performed on hydrogels with carbon nanotubes and focus on electrical enhancement of hydrogels.\textsuperscript{34,199}

The first of the studies focused on gelatine methacrylate (gelMA) hydrogel hybrids with carbon nanotubes (CNTs).\textsuperscript{199} In the study, carbon nanotubes were aligned throughout the hydrogel under dielectrophoresis force, as described in the Figure 8. The method uses two platinum electrodes to induce electric field in the material. The material successfully yielded voltage output from the hydrogel. Additionally the study successfully incorporated muscle cells into the hydrogel, therefore creating a material for fabrication of contractile muscle myofibrils that can conduct electricity. Aligned CNTs in GelMA hydrogels showed higher conductivity compared with randomly distributed CNTs in the GelMA hydrogel and the pristine GelMA hydrogel. Due to the high electrical conductivity of aligned GelMA-CNT hydrogels, the engineered myofibers cultivated on these materials demonstrated more maturation and contractility, particularly after applying electric stimulation along with CNT alignment, compared with the corresponding muscle myofibers cultured on pristine GelMA or GelMA hydrogels with randomly distributed CNTs. In summary, a dielectrophoretical method was proposed to achieve highly aligned CNTs within GelMA hydrogels in a facile and rapid way. Such GelMA-CNTs hydrogels with tuneable mechanical and electrical properties can be effectively used in biosensing, development of electrically responsive tissue scaffolds and for various other biomedical applications. Hence this method might be used to make micro-patterns of CNTs inside the biological scaffolds for various biological applications. Shin et al. went a step further in formulating electrically conductive GelMA hydrogels and dispersed highly conductive rGO instead of CNTs.\textsuperscript{200} GO and rGO are known to have lower cytotoxicity compared to CNTs.\textsuperscript{201,202} The engineered cardiac tissue constructs are thought to potentially provide high affinity tissue models for drug delivery/screening studies and the investigations of cardiac tissue development and/or disease processes \textit{in vitro}. It is desired to form soft - molecular permeable electronic devices for electrical stimulation and recording of electrical signals in living tissues, either \textit{in vivo} or \textit{in vitro}. 
Figure 8: Process of CNT alignment as bundles within the GelMA hydrogel under dielectrophoresis (DEP) force. a) Schematic representation of the fabrication process for CNT alignment within the GelMA hydrogel. Dispersed CNTs’ in the GelMA pre-polymer were introduced into the 50-μm height chamber and patterned by DEP forces (20 V and 2 MHz) within the electrodes. The chamber was then irradiated with UV light for 150 s. b) Phase contrast images of the CNT alignment over time. CNTs were aligned after 20 seconds. c) Length of CNT bundles over time. Scale bar shows 50 μm. (Reproduced with permissions from 199, Wiley).

Another study of electrical response using CNTs was performed on peptide hydrogels of 8 amino acids long (FEFKFEFK).34 In this study, Scanning Probe Microscopy (SPM) examination of mica surfaces modified with F8 peptide (FEFKFEFK) alone and with F8 and single wall CNTs (SWCNTs) together indicated that this peptide can wrap around the CNTs. This allowed the hybrid to become hydrophilic and enabled dispersion
of this material in aqueous media. For the first time, modified nano-mechanical measurements during atomic force microscopy (AFM) examination of the peptides SWCNT samples have been successfully used to distinguish peptide fibres from SWCNTs. The electric force microscopy (EFM) technique, which is a type of dynamic non-contact atomic force microscopy, where the electrostatic force is probed, was successfully applied to identify SWCNTs and distinguish them from self-assembled F8 peptide fibres. Finally the study showed that adding salt to F8–SWCNT dispersions yielded formation of hybrid F8–SWCNT hydrogels. This study highlighted the importance of studying interactions between the native material and the nano-filler added. It will be further discussed in chapter 3 in relation to our work on graphene-based materials interacting with FEFKFEFK peptide.

Another research group has looked into using the aromatic peptide of diphenylalanine with graphene and synthesised nanowires made of graphene. This was done by dropping a peptide solution of diphenylalanine in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) into an aqueous graphene dispersion under mild mechanical shaking. Under optimal assembly conditions, almost all reduced graphene and diphenylalanine participated in the core/shell assembly, leaving a transparent aqueous solvent. The peptide was then calcinated leaving a hollow graphene-shell network. Whilst the network was actually used by the authors to produce a supercapacitor, its nature is very similar to that of electro conductive hydrogels and might be potentially used for forming nanoelectronics devices for biomedical applications.

Mezzenga et al. have also presented an original method for the preparation of bio-inspired, water-based and biocompatible conductive biopolymer nanocomposites with very well-dispersed graphene within the gelatin matrix. The authors prepared the rGO/gelatin nanocomposites based on ionic interaction between negatively charged GO and positively charged gelatin chains and then following with an in situ reduction of GO at 95 °C in the presence of ascorbic acid and gelatin under vigorous stirring. Then, they prepared films by mixing the obtained gelatin/graphene dispersion with extra gelatin in order to obtain the desired graphene concentration, and then solvent casted the final mixture. The electrical properties of these nanocomposites were comparable to the best values reported in the literature, but with a percolation threshold significantly lower than the lowest values ever reported for graphene nanocomposites (0.06 vol%), and for conductive biopolymer nanocomposites (0.15 vol%). These findings could open further doors to the search of cheap optically transparent electrodes and due to the water adsorption features of gelatin
matrices, be used as biosensors or stimuli-responsive systems trigger by electric field variations.

1.10 Graphene and cell signalling

Graphene-supported scaffolds have been used to study cell twitching and signalling as well as biomolecule sensors. Cohen-Karni et al. used single layer graphene field effect transistors (G-FET) alone and simultaneously with silicon nanowire FET (SiNW-FET) to demonstrate for the first time the recording of electrogenic cells. Extracellular signals with signal to noise ratio greater than 4 were observed from G-FET conductance signals recorded from spontaneously beating embryonic chicken cardiomyocytes. Such devices have great potential in real-time tracking of cell functionality when coupled with tissue-engineered scaffolds.

Another interesting study developed functionalized GO chips for highly sensitive capture of circulating tumor cells (CTCs) in the blood of cancer patients. CTCs are responsible for the spread of cancer to secondary sites leading to development of metastases, a major cause of the mortality in cancer patients. Hence, isolation of these cells is vital to prevent tumour metastasis. An effective method to isolate CTCs with high sensitivity and low target cell concentration from the blood of pancreatic, breast and lung cancer patients was developed by using phospholipid–polyethyleneglyco-amine (PL–PEG–NH₂) functionalized GO nanosheets on a patterned gold surface. Such studies demonstrate a significant accomplishment towards development of graphene-based diagnostic chips that can eventually be used as sensors for targeting biomolecules from patient samples. Furthermore, cell behaviour was shown to be responsive to rGO by Wu et al. They formed a few-layer reduced graphene oxide (frGO) films and controlled the reduction level and surface oxygen content. The obtained results indicated the strong influence of oxidation levels on cellular behaviour, with the best performance for cell attachment, proliferation and phenotype being obtained in moderately reduced frGO. It was observed that cell performance decreased significantly with the increased level of thermal reduction. These results emphasize the important role of surface physicochemical characteristics of graphene and graphene-based materials in their interactions with bio-components. In particular, the role of active biomolecules adsorption and their usefulness in various biomedical and bio-electronic applications.
1.11 High adsorption of biomolecules to graphene and its biological properties

As previously mentioned, graphene and its derivatives, due to their large surface area hold a great potential for adsorption of active biomolecules. Also, materials used for biosensing purposes may not necessarily be formed by means of covalent chemical linking. On the contrary, very recently Li et al. showed an amyloid-GO immobilization platform, in which a GO and lyzosome hybrid electrostatically held Au nanoparticle catalysts and enzymes for improved glucose-sensing activity.208 The catalytic properties of both Au nanoparticles and enzymes were not disturbed by the presence of GO and lyzosome fibrils. This worked showed remarkable properties of GO for adsorption of biological molecules and proved that they retained their bio-activity even when adsorbed. Indeed, the property of graphene of exhibiting strong electrostatic and π-π stacking interactions with other materials (in particular biological) may lead to two possibilities. Either these materials can be used to inhibit growth of particular structures by adsorbing unwanted molecules from the surroundings (for example, inhibiting peptide plaque formation in Alzheimer’s disease) or initially molecules can attach to graphene that will trigger particular action in the environment.

1.12 Interactions with amyloid fibrillar structure

Mezzenga et al. have also performed studies on interactions between carbon nanomaterials with amyloid fibres.209,210 Firstly, they have presented new hybrid hydrogels based on β-lactoglobulin amyloid fibrils and sulfonated multiwalled carbon nanotubes (MWNTs).209 In their study, they functionalized surface of MWNTs with sulfonic functional groups covalently using a mild and environmentally friendly diazonium reaction and by a physical π–π interactions and pyrene sulfonic acid. The obtained hydrogels show reversible pH-response: i.e., gelling at acidic pH lower that the isoelectric point of amyloid fibrils, and liquid state for larger pH values. This study shows very important interactions between carbon nanomaterials and amyloid-like structures which are built mainly from β-sheets and indicate that hybrid hydrogels with responsive and new physical, biological or chemical properties can be formed for biological applications, drug release, sensors, and tissue engineering.

In the follow-up studies, Mezzenga et al. looked at the interactions between amyloid fibrils and graphene and their subsequent combination for formation of
biodegradable composite materials with adaptable properties.\textsuperscript{210} This study shows that positively charged amyloid fibrils can generate an overall positive surface charge density when adsorbed onto graphene nanosheets (electrophoretic mobility $1.5 \, \mu\text{m.cm.V}^{-1}.\text{s}^{-1}$), leading to stable graphene–amyloid fibril colloidal dispersions. Without amyloid fibrils, the reduced graphene was not stable and aggregated within a few seconds after stirring due to strong $\pi-\pi$ stacking and hydrophobic interactions. This new material was shown to be highly conductive due to presence of rGO flakes. It was also shown to be degradable by enzymes. Furthermore, it can reversibly change shape in response to discrepancies in humidity, and can be used in the design of biosensors for quantifying the activity of enzymes, with its properties being tunable simply by changing the graphene-amyloid ratio.

\textbf{Figure 9:} Schematic illustration of GO modulating the Aβ33–42 peptide assembled structure. a) Mature fibrils were formed in the absence of GO, whereas b) short fibrils and c) films were formed in the presence of GO.

Mahmoudi \textit{et al}. reported the protective role of GO and protein-coated GO surfaces in amyloid-β fibrillation process, which is involved in various neurodegenerative disorders.\textsuperscript{211} It was attributed to the large surface area of GO leading to adsorption of amyloid monomers, thus delaying fibrillation process. Interestingly, a similar report showed interactions between amyloid peptides and graphene oxide.\textsuperscript{120} This study used GO
as the modulator for tuning the formation and development of amyloid fibrils (Aβ33–42). Similarly to Mahmoudi, by using AFM temporal evolution measurements, the group revealed that the initial binding between the peptide monomer and the large available surface of the GO sheets can redirect the assembly pathway of amyloid-β fibrils. In the study, a film on the surface of GO formed, instead of a fibrillar network at higher concentrations of GO (>5 µg.mL⁻¹), as shown in Figure 9. These interactions between β-sheet like amyloid peptides and the graphene surface will be further discussed in chapter 3 and crucial for the design of hybrid hydrogels with tailorable physicochemical properties.

1.12 Mechanical properties of graphene in tissue engineering

In 2006, the seminal work by Engler et al. showed the effect of substrate stiffness on the differentiation of MSCs into different cell lineages. In particular, three polyacrylamide matrices were prepared with Young’s moduli of 0.1-1 kPa, 8-17 kPa and 25-40 kPa, respectively. These induced differentiation of stem cells into brain, muscle and bone lineages, respectively, suggesting that stiffness of materials is the main cause of cell differentiation. The high Young’s modulus of graphene (~1 TPa) can therefore be used as a nano-filler that tunes the properties of composites in order to induce specific biological response. In fact, graphene-based materials of varied mechanical properties have been explored for wound healing, stem cell engineering, regenerative medicine and tissue engineering. Although hydrogels have viscoelastic and transport properties mimicking natural tissues, their weak mechanical properties can limit their use in many tissue engineering applications. Graphene has excellent mechanical properties (high elasticity, strength, flexibility) and the ability to tailor various functionalities on flat surfaces. Hence, it can be potentially used as a reinforcement material in hydrogels, biodegradable films, electro-spun fibers and other tissue engineering scaffolds. Indeed, incorporation of GO into polyvinyl acetate (PVA)-based hydrogels, significantly enhanced tensile strength (132%) and compressive strength (36%) of composite hydrogel soft solids without affecting their cytocompatibility. Furthermore, graphene-reinforced chitosan films showed enhanced mechanical properties while not showing toxicity when tested on murine fibrosarcoma L929 cell culture. GO-chitosan hydrogel scaffolds prepared by covalent linkage of chitosan amino groups with carboxylate groups of graphene oxide exhibited significant improvement in cell adhesion, differentiation proliferation and calcium phosphate deposition by mouse pre-osteoblast
MC3T3-E1 cells. Although graphene sheets are non-biodegradable materials, the low graphene content in graphene/chitosan composites may limit any possible negative influence of graphene on cells after chitosan has decomposed in the body. This area of research is currently being tested and scientists wait for further results on the degradability of graphene, which intrinsically will depend on the type and size of graphene material used. Graphene/chitosan scaffolds retain their size and shape under physiological and extreme pH conditions as compared to those of chitosan alone.222

1.13 How all properties of graphene can move stem cell engineering forward

Besides wound healing, a number of studies have been performed exploring the use of graphene for stem cell engineering143, 214-216 and musculoskeletal tissue engineering.223 Chen et al. performed studies on graphene and GO platforms for proliferation and differentiation of induced pluripotent stem cells (iPSCs).214 They observed that just graphene surfaces support iPSC culture and allow for spontaneous differentiation. As compared to glass, GO showed faster iPSC proliferation and endodermal differentiation whereas graphene exhibited proliferation comparable to glass and suppressed the endodermal differentiation. Thus, GO-coated scaffolds may be used to direct iPSC differentiation into endodermal lineages (hepatocytes and insulin-producing β cells) whereas graphene-coated surfaces can be used for subculture and expansion of iPSCs as it maintains their pluripotency.

Graphene coated surfaces with varying stiffness and roughness have also been evaluated for differentiation of human mesenchymal stem cells (hMSCs) and pre-osteoblasts into osteoblasts. G and GO-coated surfaces exhibited accelerated cell adhesion, proliferation and differentiation of hMSCs as compared to those cultured on polydimethylsiloxane (PDMS), polyethylene terephthalate (PET), glass, and Si/SiO₂ substrates.143 When cultured in osteogenic medium, graphene remarkably accelerated the differentiation of hMSCs in a rat, which is comparable to the presence of bone morphogenic protein-2 (BMP-2) on uncoated surfaces.215

Chung et al. have also investigated the effects of GO-coated substrates on the fate of human adipose-derived stem cells (hASCs).224 As others have already shown, nanoscale topography of artificial substrates does greatly influence the fate of stem cells including
adhesion, proliferation, and differentiation. Chung et al. introduced GO film as an efficient platform for modulating structure and function of hASCs. GO was deposited on glass substrate using a self-assembly method, where the substrate was simply immersed in the GO solution (2 mg.mL⁻¹) and GO films formed. The hASCs grown on the GO films showed increased adhesion, indicated by a large number of focal adhesions, and higher correlation between the orientations of actin filaments and vinculin bands compared to hASCs grown on the glass (uncoated GO) substrate. It was also found that the hASCs showed the stronger affinity for GO films than the glass. Interestingly, the GO film enhanced differentiation of hASCs including osteogenesis, adipogenesis, and epithelial genesis, while chondrogenic differentiation of hASCs was decreased, compared to tissue culture polystyrene as a control substrate.

Application of graphene-based materials in musculoskeletal tissue engineering has also been explored using mouse myoblast C2C12 cell lines. GO showed higher myotube fusion/maturation index and upregulated expression of myogenic genes (MyoD, myogenin, troponin T and myosin heavy chain) compared to rGO. The gene regulation is linked to surface roughness and surface oxygen content on the GO/rGO, which influence adsorption of serum proteins. The adsorbed proteins are believed to cause enhanced cellular behaviour, when present on graphene derivatives in comparison to their free form in solution. In another study, fully fluorinated graphene induced higher proliferation of MSCs promoting neuronal differentiation in comparison to non-fluorinated graphene. This was further enhanced when MSCs were confined into micro-channels patterned onto fluorinated graphene in the absence of any chemical inducers.

The studies reported above, and many more were performed on 2D graphene-coated substrates. Whilst many people report the positive effects of their chosen substrates, physical interactions are often omitted. Wang et al. have looked at this issue by chemically functionalizing carboxylated GO with different surface charge groups: amino- (–NH₂), poly-m-aminobenzene sulfonic acid- (–NH₂–SO₃H), and methoxyl- (–OCH₃). GO charge was measured using ζ-potential and neutral, zwitterionic, or negatively charged graphene oxides were confirmed to be fabricated. Surprisingly, positively charged GO with ζ-potential value of 40.4 mV (functionalized with NH₂ groups) was found to be more beneficial for neurite outgrowth and branching. This study highlights the importance of understanding the physical interactions in biomaterials and substrates for biological
purposes and emphasizes the possibilities of modulating biological responses by both chemical and physical approaches.

2D materials need to be further developed in order to mimic a functional 3D extracellular environment. Indeed, recent advances in the field include utilization of graphene foams as three-dimensional (3D) scaffolds for neural stem cell (NSC) culture and human stem cell differentiation. 3D graphene foam (GF) was developed and used for human stem cell differentiation. 3D GFs supported the attachment and viability of hMSCs, and induced spontaneous osteogenic differentiation. These results have drawn the attention of many scientists to the possibility of developing graphene-based strategies for osteogenic and conductive tissue engineered constructs. The fabrication of 3D GFs was presented as a low cost and developed approach which was found to be highly scalable to bone tissue sizes. Culturing readily available hMSCs in these 3D constructs, along with their potential for multilineage differentiation, holds great promise for novel, advanced strategies in regenerative medicine. Similar 3D-GFs were shown to act as robust scaffolds for NSC culture in vitro. It was found that 3D-GFs supported NSC growth and also kept cells at a more active proliferation state with upregulation of Ki67 expression than that of 2D graphene films. Meanwhile, 3D-GFs can enhance the NSC differentiation towards astrocytes and neurons. Furthermore, 3D-GFs were shown to be an efficient conductive platform to mediate electrical stimulation for differentiated NSCs. The fabrication of these 3D GFs was performed using nickel foam template precursors. Whilst the efficiency of the scaffolds is promising, the incorporation of Ni alloys into human body may have potential side effects and biodegradability is compromised. Therefore others have focused on more bio-friendly approaches. For example, Shin et al. fabricated 3D composite scaffolds using gelatin methacrylate (GelMa) and GO. Incorporation of GO into GelMa hydrogels enhanced their mechanical and electrical properties with no adverse effect on encapsulated fibroblast cells, which highlights the potential of use of GO as a nano-filler in hydrogels for 3D cell culture growth applications and engineering of functional tissue constructs.

Advanced materials that are highly biocompatible and easily modifiable with biomolecules are of great importance for bio-interfacing and the development of biodevices. Recently, a biocompatible conducting polymer based nanocomposite was electrochemically synthesized through the electropolymerization of poly (3,4-ethylene
dioxythiophene) (PEDOT) in the presence of GO as the only dopant. GO contains many negatively charged carboxyl functional groups and is highly dispersible in aqueous solution, enabling its facile incorporation and even distribution throughout the conducting polymer. PEDOT/GO films exhibited minimal cytotoxicity after 24 h and supported neuron growth with significantly longer neurites than a control poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT/PSS) film, indicating that the PEDOT/GO film provides a positive growth signal to developing neurons. The negatively charged groups exposed freely on the surface of the nanocomposite PEDOT/GO allowed easy functionalization with biomolecules. Functional laminin, a peptide, RNIAEIIKDI (p20), was covalently linked to the surface of the PEDOT/GO film and maintained its bioactivity, as evidenced by an increased neurite outgrowth from neurons cultured on the functionalized composite surface. This is another example of simple functionalization that efficiently increased the biological function of the material. Bio-functionalized PEDOT/GO nanocomposite, along with its low electrochemical impedance, minimal toxicity and permissiveness to neuron growth, shows high potential as a material for widespread biosensing, neural interfacing and other tissue engineering applications.
1.9 Study Aim and Outline

From the previous literature, it is evident that progress in biomaterial and tissue engineering fields requires the design of novel functional biomaterials, in particular, functional three dimensional (3D) scaffolds that serve as an extracellular matrix for cell proliferation, adhesion and friendly host environment. The materials used must be 1) biocompatible – to avoid inflammation and negative immune response from the organism, 2) mechanically tuneable – to match the exact properties of tissues that the material will be used for, 3) offer further attractive physicochemical properties for drug delivery and bio-functionalization, and 4) be reproducible, reliable and cost-effective.

Self-assembling β-sheet peptide hydrogels have been shown to hold potential for the construction of soft materials that have all the necessary properties. The primary structure of peptides is easily adjustable, and the overall structure can be tuned by controlling peptide concentration and the sequence of amino acids. They can be designed to be stable under physiological conditions such as pH, ionic strength, temperature, light intensity or presence of enzymes. Overall intrinsic interactions (electrostatic, hydrophobic), sequence length, and replacement of amino acids have been shown to have a great effect on the self-assembly process and secondary structures of peptides. Whilst a lot of research has been done and applications are emerging for these types of materials, the links between the physiochemical properties of the peptides and the materials performance (i.e., for example its shear thinning behaviour) is not yet fully understood. Chapter 2 describes the relationship between peptide molecular structure and the formed hydrogel and puts emphasis on the importance of understanding the material response to shear. For this part of work, four peptides: FEFKFEFK (F8), FKFEFKFK (FK), KFEFKFEFK (KF8) and KFEFKFEFKK (KF8K) (F – phenylalanine, E – glutamic acid, K – lysine) were rationally designed and used at identical charge to explore the effect of lysine rich β-sheet self-assembling sequences on the shear thinning behaviour and final properties of bulk hydrogels. By varying peptide sequence design, concentration of the peptide, the aggregation tendency of the nanofibres formed and the balance of nanofibre junction strength versus fibre cohesive strength, the existing theory of the shear thinning behaviour of this branch of materials was extended.
The incorporation of nano-fillers for forming hybrid materials was also shown in the literature. These materials were developed to form 2D and 3D biologically responsive scaffolds. Whilst the bio-functionality is a hot area and focus of studies, there is still a large gap in understanding how to design materials and manipulate governing principles of interactions between the nano-filler and the host material to achieve the desired goal. Deeper analysis of such interactions between the host material and carbon nanomaterials will be demonstrated in chapter 3 using β-sheet nanofibres to form the 3D hydrogel network (host material) and various nano-fillers (graphene-based materials). In particular, the insights into understanding of basic interactions between the two materials will be given.

Interestingly, investigations of alignment of nanofibres in chapter 2 shed new light on the possibilities of investigation of 2D liquid crystal graphene-based materials in terms of their shear behaviour. Shear-induced control of alignment of GOLCs would be of high advantage to the field and could lead to new, exciting applications. The thorough investigation was therefore done on 2D GOLCs and will be presented in chapter 4. In particular, the combinatorial technique using rheometry and polarized light imaging (Rheo-SIPLI) and Rheo-SAXS were used in various geometries to probe in-situ the formation of new structures of sheared GOLCs. In particular, polarized light images indicated simultaneous formation of Maltese cross and shear banding for GO under wide range of shear rates used, which has not been previously observed for GOLCs.

Finally, Chapter 5 contains a short summary of all the major findings coming from this research, as well as short discussion of its implication in the field of advanced materials and suggestions for the future work.
Chapter 2: Shear thinning properties of β-sheet nanofibrillar peptide hydrogels

The work in this chapter was supervised by Prof. Alberto Saiani and the outcome from this chapter will be submitted to *Journal of American Chemical Society (JACS)* under the title of:

**Sheet edge interactions in β-sheet self-assembling peptide hydrogels: slippy fibres vs. sticky fibres**

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Author Contributions
A. Smith performed TEM imaging. J.K.W. prepared all samples and conceived all other experiments. O.O.M. contributed to the SIPLI measurements. J.K.W., A. Smith and A. Saiani co-wrote the manuscript and analysed the data. All authors commented the paper.
Abstract

Shear thinning peptide hydrogels have attracted wide interest due to their potential use in tissue engineering and biomedical applications as 3D scaffolds. The relationship between peptide molecular structure and the formed hydrogel is important to understand the material response to shear. In particular, the physicochemical properties of peptide based biomaterials will affect the feasibility of injecting them during medical procedures. Four peptides: FEFKFEFK (F8), FKFEFKFK (FK), KFEFKFEFK (KF8) and KFEFKFEFKK (KF8K) (F – phenylalanine, E – glutamic acid, K – lysine) were rationally designed and used at identical charge to explore the effect of lysine rich β-sheet self-assembling sequences on the shear thinning behaviour. Titration experiments yielded shifts in apparent pKa values of charged groups in the peptide sequence due to self-assembly. This in combination with state phase diagrams allowed selection of pH values for each of the peptide corresponding to identical charge state for direct comparison. In this chapter the shear thinning behaviour of these peptides was shown to be affected by the peptide sequence design, concentration of the hydrogel, the aggregation tendency of the nanofibres formed as well as the balance of nanofibre junction strength versus fibre cohesive strength. Results of structural analysis during the shear flow show three distinct possible states: alignment of fibres, alignment of fractured gel networks (domains > 200 nm) and disordered state, thereby extending the existing theory of the shear thinning behaviour of this type of material.

Keywords: Peptide, nanofibre network, hydrogel, β-sheet; self-assembly, shear moduli
2.1 Introduction

β-sheet peptides that can self-assemble into nanofibres and form hydrogels are of high interest for the development of materials due to their potential use in biomedical and tissue engineering. These applications were already previously discussed in chapter 1. The primary structure of peptides is easily adjustable, which allows having control over structure and mechanical properties. Therefore interchanging amino acids in the defined peptide sequence and the peptide sequence length itself will affect the final properties of a peptide hydrogel.

From the literature review (chapter 1) it is evident that by controlling peptide concentration, and the distribution and sequence of amino acids, the resulting properties of the hydrogels can be tuned. The effect of charge state of the peptide, electrostatic interactions, pH change and ionic interactions are known to significantly affect the self-assembly process. A range of stimuli, which include: pH, salt concentration, light, temperature or presence of enzymes can therefore be used to control the self-assembly process and tune the resulting properties of the formed hydrogels.

Replacing amino acids or changing the length of peptide will affect the intrinsic forces (electrostatic, hydrophobic, hydrogen bonding), thus affecting the mechanical properties of formed hydrogels. The rheology of the β-sheet peptide hydrogels usually indicates $G'$ (stiffness) values in the 100 Pa to 10 MPa range. The difference in elastic modulus comes from differences in assembly kinetics, in particular increased fibrillar branching and physical cross-links (junctions) rather than a change in the fibril nanostructure or persistence length. In particular, using spin echo neutron scattering, Schneider et al. showed that increased scattering for one of their peptides indicates a heterogeneous network with a mesh size tighter than that of the other peptide, where a lysine was changed to glutamic acid. This single amino acid change in the primary structure led to a 5-fold increase in $G'$ value of the hydrogels formed. This one amino acid substitution changed the distribution of charge on a peptide sequence used and led to a change in electrostatic interactions between the individual peptides. This affected the self-assembly process and subsequent interactions between formed nanofibres. This had implications in the overall nanofibrillar network formed, and to the changed storage modulus of the bulk hydrogel.
**Figure 10:** Mechanism of shear thinning of β-sheet peptide hydrogels. Upon shear, the hydrogel formed from nanofibrillar network can be: a) broken into domains that allow hydrogel flow. Immediately upon shear release, the domains reform a bulk hydrogel network leading to a soft solid hydrogel recovery; b) disentangled into individual nanofibres that align along the flow (shear) flow direction.

β-sheet peptide hydrogels are typically designed to be injectable. During the injection procedure, nanofibrillar peptide hydrogels will experience shear forces. Therefore, the viscoelastic properties, in particular shear-thinning and recovery mechanisms of such hydrogels should be well-defined. Schneider and Pochan propose that breaking of the hydrogels under shear results in small isotropic disconnected aggregates of self-assembled nanofibres of sizes > 200 nm. Under shear, the gel network is fractured into domains that allow the gel to flow (Figure 10a). Once the shear has stopped, the hydrogel domains immediately percolate into a network leading to the immediate recovery of a solid hydrogel. Interestingly, Schneider and Pochan show that hydrogel rigidity recovers close to values prior to shear via nanofibrillar network relaxation at the boundaries between previously fractured domains. However, this mechanism would only
work if the attraction force (junction strength) between two adjacent nanofibres in nanofibrillar network is stronger than cohesive forces keeping nanofibre intact (cohesive strength). In general, the forces that keep the nanofibre intact are mainly hydrogen bonds (which act along the fibre, as previously shown in chapter 1). For particular molecular designs of nanofibres, the second mechanism of deformation of the nanofibrillar network under shear can occur. In particular, if the junction strength between nanofibres is lower than cohesive strength holding nanofibre (hydrogen bonds), then under shear, nanofibres can experience sliding, disentanglement and eventual alignment (Figure 10b). The aim of this chapter is to explore the difference between the two shear-thinning mechanisms using β-sheet peptides that form nanofibrillar hydrogels.

2.2 Results and Discussion

2.2.1 Self-assembly behaviour

In this chapter four peptides: FEFKEFK (F8), FKFEFKFK (FK), KFEFKFEFK (KF8) and KFEFKFEFKK (KF8K) (F − phenylalanine, E − glutamic acid, K − lysine), which are based on the alternation of hydrophobic and hydrophilic residues originally developed by Zhang and co-workers were designed (Figure 11)84, 230 These sequences allow formation of β-sheet fibrils that form nanofibres with the same hydrophobic core from four phenylalanines, but with different distributions of hydrophilic amino acids: E and K (Figure 11). Initially, the self-assembly process of F8, FK, KF8 and KF8K will be investigated in relation to peptide concentration and pH and will be correlated to charge on the peptide. These were explored using titration experiments for a series of difference concentrations of all peptides. This resulted in 1) titration curve of peptide solutions at 1 mg.mL⁻¹ concentrations and 2) the phase diagram for each peptide showing the physical state of the sample as a function of concentration and pH.

All of designed peptides contain the glutamic acid (E), lysine (K), C- and N-terminus which can be charged depending on the pH (Table 1). Furthermore, the hydrophobicity of glutamic acid and lysine changes depending on the pH (Table 1).
Figure 11: Schematic representation of the cross sectional area for four designed β-sheet forming peptide nanofibres: a) F8, b) FK, c) KF8 and d) KF8K. These sequences minimize contact between water and the hydrophobic faces by pairing to form β-sheet fibres with the same hydrophobic residue side chains hidden in the fibre core, but surrounded by different hydrophilic outer part rich in glutamic acids and lysines. (F – phenylalanine, E – glutamic acid, K – lysine).

Table 1: pKa and hydrophobicity (H) values of charge residues on the peptides. The hydrophobicity values in the table below are normalized so that the most hydrophobic residue is given a value of 100 relative to glycine, which is considered neutral (0 value). The scales were extrapolated to residues which are more hydrophilic than glycine^{231, 232}. Introduction of more lysines increases hydrophilicity of the peptide nanofibre.

<table>
<thead>
<tr>
<th>pKa value of side chain group</th>
<th>H (pH 2)</th>
<th>H (pH 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic Acid (E, COOH)</td>
<td>4.25</td>
<td>8</td>
</tr>
<tr>
<td>Lysine (K, NH₂)</td>
<td>10.53</td>
<td>-37</td>
</tr>
<tr>
<td>C-terminus (COOH)</td>
<td>2.18</td>
<td></td>
</tr>
<tr>
<td>N-terminus (NH₂)</td>
<td>9.13</td>
<td></td>
</tr>
</tbody>
</table>
The theoretical charge on each peptide was calculated using Equation 1 (chapter 1) and is shown in Figure 12a. To compare the experimental charge states with the theoretically calculated values, titration experiments were performed using NaOH (Figure 12b). Glutamic acid and lysine side chains together with C- and N-terminus, become deprotonated during titration with the addition of NaOH. If the monomer peptides were free in solution and only undergo the titration of the charge groups upon addition of NaOH it would be expected that their NaOH/pH titration profiles would match the theoretical charge/pH profiles. However, self-assembly and the charge of neighbouring amino acids in a peptide sequence can cause an apparent shift in the pKa values of charge groups, and this will result in the NaOH/pH titration profiles deviating from the theoretical profiles. Therefore, titration experiments were performed on samples at concentrations of 1 mg.mL\(^{-1}\), at which peptides exists only in the fibrillar structure, but do not aggregate to form networks and hydrogels, as previously shown by Aggeli et al.\(^9\) At such low concentrations, no precipitation was observed for any peptides.

**Figure 12:** (a) Theoretical charge state on each peptide as a function of pH. Horizontal lines depict pH regions for isoelectric point (0 e\(^-\)) and +2 e\(^-\) charge at which typically self-assembling peptides are forming transparent hydrogels. (b) Molar ratio of added NaOH solution to the peptide (starting concentration: 1 mg.mL\(^{-1}\)) solution as a function of pH. The experimental titrations show small shifts in apparent pKa values in all peptides as compared to the theoretical charge calculations. Grey vertical lines represent the theoretical pKa values of charged groups as indicated in Table 1.
The titration profile of F8 peptide matches the theoretical charge profile of this peptide. Nevertheless, titration results for FK, KF8 and KF8K reveal the theoretical pKa values for N-terminus, glutamic acid (E) and lysine (K) side chains in self-assembling peptides were shifted, which is most likely due to the effect of neighbouring amino acids. In particular, the distribution of charges on the peptide will have an effect on the overall titration profile. This is clearly seen for the KF8K peptide, for which a shift in the pKa value of the N-terminus was observed (Figure 12b). This was most likely caused by the strong electrostatic repulsion from neighbouring positively charged lysines (at pH < pKaK) in the structure of this peptide (Figure 11d). In general, the charge of a given amino acid will have a strong effect on the pKa values of the neighbouring amino acids. For example, it will be harder to deprotonated a COOH group on a side chain of E placed in the surrounding of three lysines (FK) in comparison to surrounding of two lysines (F8). Therefore, the actual pKa value of this amino acid should increase, which is indeed the observed case for FK peptide (Figure 12b).

To further extend understanding of the self-assembly of the four chosen peptides, a range of peptide samples at specific concentrations and pH values were prepared and their physical state was identified (Figure 13). This also allowed the critical gelation concentration (CGC) to be identified over the pH range 2-12 for all four peptides. Four distinctive phases were observed: clear gel, cloudy gel, clear liquid and precipitate. For the peptide F8, clear and optically transparent hydrogels were observed to be formed below pH 4, and above 10.5, above CGC (Figure 13a). In the pH regions of 4 to 6.5 and 9 to 10.5, cloudy gels were observed to form. Visual cloudiness of hydrogels is caused by the peptides that are partially self-assembling and partially forming aggregates of sizes larger than 700 nm - 1 µm. Previously, Aggeli et al. reported that the change of a hydrogel from clear to cloudy occurs when overall charge of a peptide is lower than 1e⁻ (Z<|1|). Indeed, this observation correlates well with the change of the theoretical charge state on F8 peptide (Figure 12a). At pH 7, corresponding to isoelectric point (0 charge state) of this peptide, all F8 samples were found to show phase separation with the appearance of a clear liquid phase at the top and a white precipitate at the bottom of the test tube.
Figure 13: Phase diagrams for all four peptides with shown concentration as a function of pH. Four distinctive states were observed: clear gel, cloudy gel, clear liquid and precipitated state. Grey rectangles define pH areas chosen for further characterization of the shear thinning behaviour.

Similarly to F8, all other samples follow the trend of going from clear hydrogel to cloudy hydrogel (above CGC) from lower to higher pH. It is important to note that peptides FK, KF8 and KF8K did not show signs of precipitation above CGC, like peptide F8 did. Subsequent introduction of lysine amino acid in the peptide sequence (F8 → FK and KF8 (+1K) → KF8K (+2K)) resulted in increased hydrophilicity of peptides and their ability to interact with water environment. This in turn resulted in the ability of these peptides to remain in cloudy gel form above CGC even at their respective isoelectric points. Interestingly for FK peptide after pH 8, a transition from precipitation to cloudy to clear is observed as a function of concentration. This is an indication of noticeable difference in the self-assembly process. The exact reason for this different behaviour of the FK peptide is outside the scope of this thesis and is an interesting point for future work. Nevertheless, the CGC for this sequence is at least twice that for the other peptides at any
pH. This suggests that the tendency to self-assembly for FK peptides is lower and may relate to the charge distribution on this peptide. This is the only peptide that contains only a single glutamic acid in the peptide (Figure 11b), which may lead to fewer potential charge-charge interactions when compared to other three systems.

The phase diagram for KF8K peptide indicates that the transition from clear gel to cloudy gel occurs at pH 7.5 (Figure 13d). At that pH, theoretical charge on this peptide is $+2e^-$ (Figure 12a). Titration experiment on this peptide at low concentration yielded the shift in the pKa value of the N-terminus (Figure 12b), which indicates that actual charge of this peptide at pH 7.5 approaches $+1e^-$. This correlates well with the predications made by Aggeli et al. $^{90}$, who said that change of phase occurs when charge of a peptide approaches $Z=|1e^-|$.

This understanding allowed more precise choice of pH values for further shear thinning analysis. Usually hydrogels used for some biomedical purposes should remain transparent and with pH as close as possible to physiological pH (7). Therefore pH range at which F8, FK, KF8 and KF8K were prepared for further characteristics are: 3.5-3.8, 5.7-6.0, 5.9-6.1 and 6.9-7.1, respectively. At these pH values (and above CGC), all peptides formed clear and self-supporting hydrogels. These values do correlate with each peptide carrying approximately charge of $+2e^-$ and will be used throughout the rest of this chapter.

Under chosen conditions, a range of techniques was applied to investigate the properties of the formed clear hydrogels and peptide solutions at similar charge state. Fourier transform infrared spectroscopy (FTIR) was used to confirm that all the peptides self-assemble into β-sheet rich nanofibres which then further interact with each other to form entangled networks (hydrogels) above a critical gelation concentration (CGC). Network morphology and topology properties were investigated using transmission electron microscopy (TEM), atomic force microscopy (AFM) and small angle X-ray scattering (SAXS). The shear thinning properties were explored using shear induced polarized light microscopy (SIPLI) and oscillatory rheology as function of peptide concentration. Finally, peptide alignment was quantified using flow-SAXS.
2.2.2 From structure to physical properties of the hydrogels

Firstly, FTIR spectroscopy was used to determine the secondary structure adopted by the peptides in the formed hydrogels (Figure 14). Strong absorption peaks at around 1620 cm\(^{-1}\) and 1695 cm\(^{-1}\) were observed, which are indicative of an extended β-sheet secondary structure in peptides.\(^{234-236}\) The intensity of this peak is similar for all samples suggesting that a similar quantity of β-sheet structure is present in them due to the presence of the same hydrophobic core. The overall peptide length, number of amide bonds and lysines present vary from F8 to FK to KF8 to KF8K. This is reflected in changes in peaks at 1551 cm\(^{-1}\) and 1521 cm\(^{-1}\) of the amide II region corresponding mainly to in-plane N-H bending and C-N stretching vibrations on each peptide in the hydrogels.\(^{237}\)

![FTIR spectra](image)

*Figure 14: FTIR spectra obtained for all four peptide hydrogels prepared at the same concentration of 26.8 mM.*

The existence of entangled nanofibrillar networks consisting of β-sheet nanofibres in the produced hydrogels was confirmed by the AFM and TEM microscopy. Representative AFM and TEM regions of all four peptides are shown in Figure 15 and Figure 16, respectively. For all samples, individual fibres as well as lateral associations of nanofibres can be identified. These self-assembled nanofibres form dense networks, in which the individual nanofibres interact physically by electrostatic, hydrophobic and π-π interactions with the other nanofibres in the network. All the TEM and AFM images show topologies that are indicative of inter-fibre interactions, which are crucial for forming interlinked networks and further affecting the properties of bulk materials.
Height (representative of a nanofibre diameter) analysis was performed on the obtained AFM images where representative sections were measured across approximately 3 µm through regions of smallest observed nanofibres and included up to 15 nanofibres across the images. Diameters of the smallest measured nanofibres were $3.0 \pm 0.4$ nm, $2.5 \pm 0.4$ nm, $2.8 \pm 0.3$ nm and $2.3 \pm 0.4$ nm for F8, FK, KF8 and KF8K, respectively. Recently, Gao et al. discussed network topologies of FEFKFEFK vs FEFKFEFK peptide, where they discussed the tendency of association of individual nanofibres in the network.\textsuperscript{116} Indeed, for F8 sample large quantity of aggregates and junction points were observed in the TEM image (Figure 16). Whilst the thinnest nanofibres observed correlated well to these obtained from AFM image, F8 also showed associated nanofibres bundles of an average diameter 10 nm ± 2 nm. These larger nanofibres indicate that this system contains associated nanofibrillar network, which confirms recent results for this peptide.\textsuperscript{116} Such a high tendency for fibre association was not observed for the FK and KF8K peptides. The theoretical length of F8 peptide in a fully extended $\beta$-sheet conformation is estimate to be about 2.8 nm and comes from the approximate length of an extended amino acid end to end being 3.5 angstroms (multiplied by 8 for F8). The thickness comes from the X-ray diffraction patterns of amyloid $\beta$-sheet nanofibres where the distance on the cross $\beta$
diffraction pattern from side chain packing varies between 0.8 to 1.2 nm depending on the side chain size and packing. As this is the distance between the backbones one needs to add a few angstroms for the external side chains so 1.5 nm is an estimated thickness value. The obtained heights (diameters) from the AFM images for the thinnest nanofibres correlate well to the theoretical values.

**Figure 16:** TEM images obtained for all peptide samples. Courtesy of Dr. Andrew Smith. The KF8 image contains larger quantity of uranyl acetate staining in comparison to the other samples.

Small angle X-ray scattering (SAXS) was used to investigate the structure of the formed nanofibres (**Figure 17**). Typical scattering patterns are presented for the samples in the dilute regime (<CGC) in **Figure 17a**. In this regime the scattering observed is dominated by the form factor of the dilute scattering entities. As observed, all the samples present scattering patterns following ~ q⁻¹ behaviour at low q, which is typical for the scattering for long thin rod-like structures. It has been previously shown that for such objects (i.e., fibres), for qR<σ < 1 (R<σ: cross-section radius of gyration) the scattering intensity can be written as:

\[
\ln q I(q) \propto -\frac{R_\sigma}{2} q^2
\]

(2)

If the scattering observed is of the form described by the above **Equation 2**, then at low q a linear behaviour should be obtained in a ln[qI(q)] vs. q² representation. This is the case and can clearly be seen in **Figure 17b**. The cross-section radius of gyration, R<σ, of the fibres can be estimated from the slope of the linear section. Assuming that the fibres can be
modelled by a plain infinitely long cylinder, the $R_\sigma$ is related to the diameter of the fibre, $d$, through the following equation:

$$R_\sigma = \frac{d^2}{\sqrt{8}}$$  \hspace{1cm} (3)

By fitting the linear regions for the 4.5 mM samples (<CGC), nanofibre diameters ($d$) can be estimated as: 2.8 ± 0.1 nm, 2.9 ± 0.1 nm, 3.5 ± 0.1 nm and 3.7 ± 0.2 nm, for the F8, FK, KF8 and KF8K respectively. Fibre diameters were further extracted and plotted for all samples as function of tested concentrations in Figure 19c.

**Figure 17:** a) SAXS scattering pattern (log $I(q)$ vs log $q$) obtained for the samples below the CGC (4.5 mM). b) SAXS scattering pattern at low $q$ plotted in a $\ln[qI_N(q)]$ vs. $q^2$ representation. Best fits of curves linear sections are presented with the corresponding values of $R_\sigma$ extracted (see text for more details). c) Fibre diameter value as a function of concentration for all peptides. These values were extracted from SAXS measurements.

The peptides F8 and FK have similar nanofibre diameter of about 2.5 – 3 nm across all tested concentrations. The addition of one amino acid to the structure increases the theoretical length of peptide on average by 0.35 nm. Upon addition of lysine to the primary structure of F8 (F8 → KF8 → KF8K), the fibre diameter was observed to increase by 0.6
nm for KF8 and 0.8 nm for KF8K at 4.5 mM, which are in good agreement with the theoretical predictions.

FTIR indicates similar β-sheet conformation for all four samples, which in theory suggests similar intrinsic properties of formed nanofibres. TEM, AFM and SAXS show similar sizes of thinnest nanofibres in the network. However, there were differences in nanofibre aggregation and association observed. In particular, F8 peptide showed very high tendency for fibre association in TEM (Figure 16), whereas KF8K showed shorter and less bundled nanofibres in the AFM images (Figure 15). FK and KF8 peptides showed higher tendency for aggregation, association and bundling than KF8K. The changes in nanofibre association and aggregation (i.e., network topology) would be expected to influence the intrinsic physical properties of the formed 3D nanofibrillar network, in particular elasticity. For an entangled network of nanofibres the balance between junction force and cohesive fibre strength will affect the performance of the material upon the application of shear forces.

![Graph](image)

**Figure 18:** a) Shear modulus ($G'$) of all hydrogels at 1 rad.s⁻¹ obtained from the frequency sweep experiments performed at 0.2 % strain as a function of concentration. b) Log-Log plot of shear moduli ($G'$) vs. molar concentrations obtained for all hydrogels at 1 s⁻¹ obtained from the frequency sweep experiments performed at 0.2 % strain as a function of concentration. Linear regression curves were fitted using GraphPad (v 7.0). Power laws were observed and denoted in the table c) for each peptide.
Firstly, the mechanical properties of the hydrogels were investigated as a function of concentration using oscillatory rheometry (Figure 18a). Increasing the peptide concentrations resulted in an increase in $G'$ for each system, as expected. The F8 and FK peptides showed higher $G'$ values at concentrations above 26.8 mM compared to the other two more hydrophilic peptides (KF8 and KF8K). Both microscopy techniques suggested the higher number of junctions (i.e., aggregation and association tendency) and association of nanofibres for F8, FK and KF8K peptides. Evidently the intrinsic nanofibrillar network impacts on macroscopic physical properties of the formed hydrogels. There are numerous examples of theories that relate the intrinsic properties of polymeric fibrous objects to the storage modulus (i.e., stiffness) of the resultant material.\textsuperscript{241} One example is a theory developed by Jones \textit{et al.},\textsuperscript{242} where $G'$ of a rigid polymer networks is related to the fractal dimension $D_F$ of the objects forming the network through a power law:

\[
G' = C^{\frac{(3+D_F)}{(3-D_F)}}
\]  \hspace{1cm} (4)

where $C$ is the concentration of objects contributing to the network elasticity. The corresponding plots of log G vs log C were plotted in Figure 18b.

The power law of $C^2$ is expected for a network formed by straight (between junctions) thin fibres (with $D_F = 1$). Values of $3.4 \pm 0.9$, $4.7 \pm 0.8$, $3.3 \pm 0.4$ were obtained for F8, FK and KF8 respectively (Figure 18c). As suggested by Ramzi \textit{et al.} a higher exponent in the power law can originate from a large number of junctions which effectively lowers the concentration of objects actively participating to the elasticity of the formed network, by lowering the nominal concentration of proper rigid objects considered in this law.\textsuperscript{243} Obtained values for F8, FK and KF8 would point to higher tendency for aggregation and bundling in the nanofibrillar networks, which is in good agreement with the previously shown microscopy images (AFM and TEM). An exponent of $1.3 \pm 0.3$ was obtained for KF8K which would imply semi-flexible rods present in the system and confirms the very dissimilar behaviour of this peptide sequence, as already noted in the microscopy results.
2.2.3 Degree of alignment: sticky junctions versus slippery fibres

Recently, a method which combines rotational rheology and polarized light imaging has been developed called shear induced polarized light imaging (SIPLI)\textsuperscript{244,245} which allows a full view of a sample during rheological measurements using various geometries and different states of polarized light. It has since been applied to study the oriented lamellar phase of block copolymers\textsuperscript{246} or shear-induced crystallisation of thermoplastics\textsuperscript{244,245} and can also be applied to study orientation of liquid crystals during shear flows.\textsuperscript{244} Therefore, to investigate in detail the shear thinning behaviour of the prepared peptide hydrogels, rheology-coupled shear induced polarized light imaging (Rheo-SIPLI) was used. In used Rheo-SIPLI, the light passes through a linear polarizer and becomes polarized as depicted in Figure 27 (materials and methods). It then goes through a transparent bottom glass plate, interacts with the sample and hits the top reflective plate. The light is then reflected, goes through the sample (again), passes through the bottom transparent plate, a beam splitter, a linear analyzer crossed at 90 degrees with respect to the polarizer plane and finally reaches a CCD camera (detector). This way polarized light imaging can be performed on samples, prior to, during and after the application of shear forces. In this case the path of the polarized light goes as follows: polarized → beam splitter → sample → reflective top mirror plate → sample → beam splitter → analyzer → detector (camera). F8, FK and KF8 all showed birefringence at a shear rate $\dot{\gamma} = 1$ s\textsuperscript{-1} (Figure 19). In the SIPLI configuration, the shear flow of a sample in the tangential direction (tracing a circular trajectory around the rheometer axis of rotation) should produce a polarized light typically known as a Maltese cross (MC).\textsuperscript{244,246} It originates from the fact that light transmission is blocked in regions where the sample (here, nanofibres) is oriented parallel to either the polarizer or analyzer, and transmitted through the regions where the sample is at an intermediate angle between the polarizer and analyzer. MC pattern is commonly observed under crossed polarisers for birefringent materials in which one of the principal axes of the optical birefringence ellipsoid is always parallel to the radial direction originating from the MC centre, for example in polymer spherulites (in which polymer crystals grow radially from the nucleation centre).\textsuperscript{247} At (an edge – see materials and methods) 1 s\textsuperscript{-1} shear rate, for the F8 and FK samples below the CGC Maltese cross patterns were observed. F8 at 4.5 mM and FK at 8.9 mM formed viscous liquids (Figure 13). The observed MC clearly indicates the alignment of the nanofibres in the tangential direction to the flow. This in turn suggests that the cohesive forces (H-bonds) keeping the nanofibres intact are stronger than
the strength of junctions. For the F8, FK and KF8 peptides at concentrations above CGC the granular structure of images was observed (Figure 19). Such obtained patterns in the polarized light would indicate randomly aligned state of the sample. In this regard, the obtained images can be actually correlated to the Schneider and Pochan theory of hydrogels being broken down to small domains, which allowed the hydrogel to flow under shear\(^8\) (Figure 10a).

**Figure 19:** Polarized light images taken after 120 seconds of constant unidirectional rotational shear (at the edge) at 1 s\(^{-1}\) (see materials and methods: SIPLI) for the three samples (F8, FK and KF8) as a function of concentration. Red line represents CGC as defined previously by phase diagrams.

To further study the state of the sample under shear, all experiments were repeated at (edge) shear rate \(\dot{\gamma} = 10\) s\(^{-1}\) (Figure 20). At this shear rate, samples just above CGC started to show the distorted MC pattern (8.9 mM for F8 and KF8 and 17.6 mM for FK). It is expected that with the 100-fold increase in shear forces (\(F = ma\omega^2r\)) at the same point (\(r = \text{constant}\)) the structure of a hydrogel would break. It is evident from the Rheo-SIPLI that at higher shear rate, hydrogels must be breaking into small domains which align tangentially to the direction of the flow. It is possible that stronger forces or longer application of forces would start breaking the hydrogel domains into individual nanofibres that align parallel to each other, yet tangential to the flow. Moreover, the distortion of the
MC was found to be a function of peptide concentration. At higher concentration of peptide, there are more junctions between nanofibres, simply because of higher probability of an individual nanofibre interacting with the neighbouring one. It is worth noticing that the distortion of the MC from an ideal case (like 8.9 mM FK at both shear rates) occurred with increasing concentration. This indicates that using the same shear force, it is harder and harder to break the highly concentrated hydrogel into small domains that will align along the flow. Since many materials exhibit different properties depending on their alignment state, it will be important for biomedical researchers to carefully choose the desired hydrogel for a particular function.

Figure 20: Table of polarized light images taken after 120 seconds of constant unidirectional rotational shear (at the edge) at 10 $s^{-1}$ (see materials and methods: Rheo-SIPLI) for the three samples (F8, FK and KF8) as a function of concentration. Red line represents CGC as defined by previous phase diagrams.

All previous Rheo-SIPLI measurements were performed for 2 minutes, however, one may be interested in looking at long-term application of shear forces. Therefore, a representative F8 sample at a concentration of 17.6 mM was chosen in order to look at the alignment state after 60 minutes.
Figure 21: To analyse the obtained PLIs, the circular PLIs were transformed to Cartesian coordinates, which were further rotated by an angle of 90°. The centre of the circular PLIs is represented as bold black line on the top of the Cartesian PLIs (CPLIs). a) The CPLIs obtained at every 15 minutes of 17.6 mM F8 sample sheared (at the edge) at \( \dot{\gamma} = 10 \text{ s}^{-1} \) for 1 hour. The dashed red line represents a set of points with values of 9 s\(^{-1}\) shear rate. b) Integrated pixel intensity from region with shear rate 9 s\(^{-1}\) showing the level of alignment relating to distortion of MC. Individual plots were offset by 255 in y direction as indicated by the dashed horizontal lines embedded in the figure. From 30 minutes a better alignment state was forced. The experiments were performed using a solvent trap in the rheometer to avoid water evaporation. The centre of polar image is transformed to the first vertical line of the Cartesian coordinate picture.

In order to better represent the state of alignment of the sample, ImageJ software was used to transform polar coordinates of images to Cartesian (Figure 21a). Integrated pixel intensity from the region of shear rate \( \dot{\gamma} = 9 \text{ s}^{-1} \) (red line on the images) was performed (Figure 21b), in order to quantify the level of distortion of MC, and therefore the level of alignment. Initially, a typical state of random alignment of hydrogel domains was obtained (0 minutes). Already after 15 minutes of shear, one can observe clear appearance of a MC, especially on the edge of the image (\( \dot{\gamma} > 8 \text{ s}^{-1} \)). Then, it was clearly observed that the MC
becomes more defined as the time passes by. This is clearly visible when looking at the integrated pattern (Figure 21b), where 4 distinct peaks evolved. It is interesting to note that the clearest MC pattern started to affect alignment at lower shear rates. The level of alignment (as described by MC) was observed to be a function of time of applied shear (Figure 21a). The pattern became well-defined after 15 minutes of shear. It can be concluded from this experiment that hydrogels break into progressively smaller domains that align better under shear, if shear persists for a longer time (60 minutes in comparison to 2 minutes).

![Figure 22: a) PLIs taken after 120 seconds of constant unidirectional rotational shear (at the edge) at $\dot{\gamma} = 100$ s$^{-1}$ (see materials and methods: Rheo-SIPLI) of the KF8K as a function of concentration. No birefringence of the sample was observed at lower shear rates for the given concentrations. Optical measurements of refractive index measured for 4.5 mM of each peptide solution using ellipsometer at two angles of incidence: b) 60° and c) 65°. At any given wavelength, peptide solutions exhibit a similar optical response to that of water.](image)

The peptide KF8K did not show any birefringence at any concentration for the two previously described shear rates. Therefore a higher shear rate of $\dot{\gamma}=100$ s$^{-1}$ was used to probe alignment of KF8K at three different concentrations (Figure 22a). At this shear rate, only a higher concentration sample (26.8 mM) exhibited birefringence and formed a MC in
PLI. This suggested that the KF8K did not align at lower shear rates and a higher force than for the other three peptides was required to align this hydrogel. To confirm that the observed effect was due to alignment, optical properties of all peptide solutions were measured using ellipsometry (Figure 22b and 22c). The response for all peptide solutions across the whole spectral wavelength range was similar to that of background ddH2O (doubly distilled water). Birefringence is the optical property of a material having a refractive index that depends on the polarization and propagation direction of light. This property will intrinsically depend on the structural anisotropy of the material. Since optical properties of hydrogels do not vary significantly from those of water, it seems that the obtained PLIs for F8, FK and KF8 are due to the macroscopic aligned textures formed by the peptides. In turn, it seems that KF8K did not form macroscopic aligned structures that allowed this peptide to be birefringent. This suggests that at the molecular level, nanofibres did not interact with light in the same way as the other three peptides.

In order to explain the lack of alignment of KF8K and its lack of birefringence, one has to take a step back and look at the molecular design of the nanofibre. For this explanation, molecular models of all four peptide nanofibres in an anti-parallel arrangement were drawn (Figure 23). In the spirit of previously discussed rheological results (Figure 18) and work done by Nilsson et al.\textsuperscript{110} and Gao et al.,\textsuperscript{116} the three designed peptides F8, FK and KF8 formed ‘sticky’ junctions in the network leading to a higher aggregation tendency. These potentially originate from the hydrophobic aromatic ring of the phenylalanines close to the ends of peptides (depicted as red arrows in Figure 23). These aromatic rings can form π-π and π-cation interactions with the other peptides in fibres, thus leading to the points of high attraction, where other molecules can become physically attached. The macromolecular structure formed from these three samples exhibited birefringence upon shear and showed the changes of the structure at very low shear rate. Sticky junctions may lead to associated networks, as already discussed in the rheology section.
Figure 23: Molecular models representing a cross section through a β-sheet fibre for the four peptides: F8, FK, KF8 and KF8K. For comparison it has been assumed the phenylalanine side chains will form the hydrophobic core of the β-sheet structure reducing their contact with water. For F8, FK and KF8 (red) arrows indicate the aromatic rings coming from the phenylalanine that are exposed to water at the ends of the β-sheets, whilst (blue) arrows in the KF8K point to the hydrophobic core of the nanofibre containing all aromatic rings, fully shielded by hydrophilic amino acids that allow a water shell to form around this nanofibre to form. Courtesy of Dr Andrew Smith.

The ability of these three peptides (F8, FK and KF8) to align and their birefringence must therefore be linked to the properties of the nanofibrillar network and its topology. In contrast the last sequence, KF8K, which formed thinner, semi-flexible
nanofibres, did not show birefringence at low shear rates. In turn, it required a large force input (high shear rate) to show any birefringence and alignment. Whilst the optical properties of all four peptides remained similar, the KF8K was the only sample behaving differently to others. This change in behaviour suggested that it is related to the primary structure of this peptide. Indeed, the additional lysine residue at the end of the peptide in comparison to KF8K (as seen in the Figure 23 by blue arrows) can inhibit the ability of the buried phenylalanine aromatic ring to interact with the outer environment. Thus a fully hydrophilic outer fibre shell was formed, which could interact stronger with water molecules than other peptides. Due to its hydrophilicity, lysine can H-bond with the water molecules thereby forming a protective water shell around the nanofibres formed. Lysine is not only used in peptides and proteins to increase the hydrophilic character and solubility, but has also been shown to allow metallic nanoparticles or graphene to become dispersed in aqueous environment. Interestingly, the KF8K, which forms semi-flexible rods, must be forming very flexible networks in which the nanofibres can only interact purely by electrostatic interactions and H-bonds to form entangled networks. It appears that these links are significantly stronger than those formed via π-π stacking or π-cation in F8, FK and KF8 nanofibres. This results in the very strong hydrogel domains which can fracture at sufficiently high shear rate (\(\dot{\gamma}=100\ \text{s}^{-1}\)), but do not break easily and do not align at lower shear rates (\(\dot{\gamma}=1\) or 10 \(\text{s}^{-1}\)). This, together with the fact that KF8K has optical properties similar to water leads to a lack of birefringence.

**Table 2:** Values of pump rotation rate, volumetric flow rate and corresponding linear flow rates used in Flow-SAXS experiments.

<table>
<thead>
<tr>
<th>Pump rotation rate (rpm)</th>
<th>Volumetric flow rate (mL.s(^{-1}) = cm(^3).s(^{-1}))</th>
<th>Linear flow rate (cm.s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.087</td>
<td>11.08</td>
</tr>
<tr>
<td>40</td>
<td>0.118</td>
<td>15.02</td>
</tr>
<tr>
<td>80</td>
<td>0.155</td>
<td>19.74</td>
</tr>
</tbody>
</table>
Figure 24: Ratios of length (y) versus width (x) obtained for anisotropic 2D Flow-SAXS patterns (see Figure Figure 26) as a function of peptide concentration, presented for 3 different pump speeds (relating to flow rates: 0.087, 0.118, 0.155 mLs$^{-1}$, respectively as indicated in the Table 2). For every speed, the degree of orientation of F8 peptide is large, whereas all the other peptides at low concentrations start at low degree of orientation. As noticed, KF8K does not align at any concentration and speed used.

Recently, other groups have shown that alignment of nanofibres can be probed by a simple flow-SAXS technique and analysis of the anisotropy of the obtained 2D scattering patterns.$^{250, 251}$ Therefore, this technique was used to confirm and probe alignment of the nanofibres for all four peptides as a function of the concentration and flow rate (Figure 26, Table 2). To simply define the peptide alignment, the ratio of the length (y) and width (x) of the patterns was plotted as a function of concentration and flow rate (Figure 24). The pump rates were correlated to both volumetric and linear flow rates (Table 2), which were then translated onto shear rates for comparison with the Rheo-SIPLI measurements. Interestingly, F8 peptide exhibited the highest degree of alignment comparing to all the other peptides. In particular the y to x ratio defining alignment, at the very low concentration of F8 (2.2 mM) was always highest. Moreover, with the increase of concentration, an obvious trend of degree of alignment was noticed with F8 > KF8/FK >
KF8K (Figure 24). Indeed, the lack of alignment for KF8K for any given concentration and probed flow rate was confirmed, where the values of degree of alignment were approaching 1, which corresponds to the isotropic scattering patterns obtained (Figure 26). This confirmed that the nanofibres of KF8K peptide have significantly faster relaxation time than all the other peptides.

Finally, oscillatory rheology experiments were performed only on F8 and KF8K samples, which are representative of the two different hydrogel breaking mechanisms. These experiments were implemented to look at the recovery of peptides after simulated injection and their recovery due to cyclic applications of strain. Typical critical strain $\gamma_c$ value of these hydrogels is approximately 3-5 % (Figure 25a,b). To do this, two modes of measurements were developed. In the first one, storage modulus was observed for both samples initially for 3 minutes at 0.2 % strain. After that, both hydrogels were subject to 1000 % strain to simulate the injection. At this point, storage modulus value dropped to almost 0 and the sample was liquefied (Figure 25c). Then, the 1000 % strain was decreased back to 0.2 % and the storage modulus was observed for 120 minutes to look at the long term relaxation phase. It was clear that KF8K hydrogel recovered almost immediately (1 minute) in comparison to F8. After that, KF8K peptide hydrogel increased its original $G'$ value in 2 hours’ time to 114 % of its initial value. In comparison, it took F8 hydrogel over 100 minutes to recover to its initial state. These results support the very fast relaxation time of semi-flexible KF8K nanofibres. The next experiment was performed to look at the recovery of the two peptide hydrogels due to cyclic strain conditions. In this experiment, hydrogel samples were initially subject to 0.2 % strain for 10 minutes and then the strain was increased to 100 %. The hydrogel recovery was then monitored for 10 minutes before the cycle was repeated (6 times in total). Again, both hydrogels break at 100 %, as their storage modulus instantly drops to zero at that point (Figure 25d). In general, after 6 cycles, the storage modulus of F8 peptide hydrogel decreased by 20 %, whilst KF8K increased by 28 %. As noted in the first injection simulation experiment, the F8 peptide hydrogel recovered to 100 % in 100 minutes. In the cyclic experiments, 10 minutes were clearly insufficient for this peptide to recover. This indicates that the peptide was broken into domains that aligned. Unlike the Schneider and Pochan case, the nanofibrillar network formed from F8 nanofibres, did not immediately relax at the boundaries between the fractured domains.80 One of the reasons behind this slow relaxation may be that junction points between the F8 nanofibres are formed mainly via $\pi$-$\pi$ stacking and $\pi$-cation interactions, which usually are short range interactions. Similarly
to Schneider and Pochans’s MAX1 and MAX8 hydrogels,\textsuperscript{80} KF8K is rich in lysines. Indeed, these peptides are alike in terms of relaxation times, and no alignment was observed for them (and this work).\textsuperscript{80}

\textbf{Figure 25:} Storage and Loss modulus in an amplitude sweep (strain sweep) of a) F8, b) KF8K peptide hydrogel, prepared at 26.8 mM. Normalized $G'$ for F8 and KF8K peptide hydrogels prepared at 26.8 mM at two recovery modes: c) These measurements were monitored in time at 0.2 % applied strain for 3 minutes, then at 1000 % strain for 1 minute to simulate injection and then again at 0.2 % strain for 120 minutes. The dashed horizontal line represents 100 % of G' value (initial stiffness of a hydrogel). d) These measurements started at 0.2% strain applied for 10 minutes. After 10 minutes, strain was increased to 100% and the response was observed for another 10 minutes. This cycle was repeated 6 times in one measurement. All oscillatory rheology experiments were performed with angular speed of $\omega = 6.28\text{ rad.s}^{-1}$.

Similarly though, the nanofibrillar network made from KF8K nanofibres immediately relaxes due to its interaction with water, at the boundaries between any fractured domains. In fact, the breakage of the KF8K hydrogel may allow the nanofibrillar network to rearrange itself into a more desired topology, which enables the overall structure to be stronger than before.
2.3 Conclusions

In this chapter four self-assembling nanofibre forming peptides: FEFKFEFK (F8), FKFEEFKF (FK), KFEFKFEFK (KF8) and KFEFKFEEFKK (KF8K) have been used to demonstrate two different shear-thinning behaviours. Clear, self-supporting hydrogels, just before turning to cloudy were chosen for comparison. This corresponded with similar charge states of peptides. All formed hydrogels under these condition show nanofibrillar networks with a distinct β-sheet conformation. The difference in lysine content leads to changes in the fibre sizes and tendency of nanofibres to associate and aggregate. By applying theory developed by Jones and Marques to oscillatory rheology measurements, the network morphologies were correlated to the macroscopic mechanical properties of the formed hydrogels. The use of theory suggests that the peptide nanofibre morphologies can be divided into two groups, those with a high ability to associate and bundle (F8, FK and KF8) and others with reduced ability to form aggregates (KF8K). This association was further visualized using rheology-coupled shear induced polarized light imaging, which showed that samples that easily associated into bundles of nano-fibrous networks showed birefringence at low shear rates. KF8K peptide, which forms semi-flexible nanofibres, needed a larger shear rate (>10 fold increase) to show birefringence. Moreover, the technique allowed the macroscopic structural state to be probed upon shear. F8, FK and KF8 peptides exhibited clear Maltese cross at concentrations <CGC and distorted MC at >CGC at low shear rates. Flow-SAXS confirmed that KF8K nanofibres do not align under any probed concentrations and flow rates. Three distinct states were observed: alignment of nanofibres (for samples < CGC), alignment of nanofibrillar rich domains (> CGC, at edge shear rate of 10 s⁻¹) and a disordered state for higher concentrations of peptides or KF8K at low shear rates. Further oscillatory rheology experiments performed on representative F8 and KF8K samples confirmed the very different behaviours of these two peptides. The injection and cyclic recovery experiments clearly distinguished the junction-like topology of F8 peptide that under shear breaks into nanofibres and domains that align and slippery KF8K nanofibres that behave exactly like lysine-rich peptide MAX1 and MAX8 hydrogels. This work expands on the theory of shear thinning mechanisms of β-sheet peptide forming hydrogels and should apply to other peptides with similar sequence properties. Thus by careful primary sequence selection, concentration, and pH, the bulk physical shear thinning mechanisms can be chosen and fine-tuned for a particular application, where linear shear flow (for example, injection) is desired.
2.4 Supplementary figures

![Supplementary figures](image)

**Figure 26**: 2D SAXS patterns obtained for all four peptides at different concentrations and at linear flow rate of 19.74 cm.s\(^{-1}\). CGC defines critical gelation concentration.
2.5 Materials and methods

2.5.1 Peptides

All peptides were purchased from Biomatik (Canada). The peptides purity was confirmed using reverse phase high performance liquid chromatography (HPLC) and mass spectroscopy (MS) with typical purity > 90%. Peptides F8, FK and KF8K were purchased as TFA salt. Their salts were then replaced by HCl salts using standard procedures.\textsuperscript{252} KF8 peptide was directly purchased as the HCl salt.

2.5.2 Hydrogel Preparation

Peptides were dissolved in 400 µL or 800 µL of double deionised water (ddH\textsubscript{2}O) by sonication at 80 kHz for 60 min and left overnight in a fridge (4 °C) in this state to equilibrate. Hydrogels were then prepared the next day by adjusting the pH with 0.5 M, 1 M or 2 M NaOH to the desired value. The desired concentration was achieved by adjusting the volume with the addition of ddH\textsubscript{2}O. All the samples were prepared at room temperature.

2.5.3 Peptide titrations

The ionization behaviour of the charged residues on the four peptides (based on Arrhenius theory): AcH\textsuperscript{+} (acid) + OH\textsuperscript{-} (base) → Neutral species + H\textsubscript{2}O (water) was investigated.\textsuperscript{253} Titrations were performed adding aqueous 0.05 M NaOH in 5 µL steps to 1 mL solution of dissolved peptide in ddH\textsubscript{2}O, with starting concentration of peptide 1 mg.mL\textsuperscript{-1}. To ensure validity of results, every time NaOH was added, the samples were vortexed for 45 seconds. Experiments were repeated twice.

2.5.4 Hydrogel phase state

Phase diagrams were determined by observation of the sample state on the third day of incubating hydrogels in the fridge (4 °C) after the preparation. Sample was
classified as a liquid when it flowed freely and as a gel when it was self-supporting upon inversion of the vial. Each experiment was repeated twice.

2.5.5 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR measurements were performed on a Bruker Vertex 80 FTIR spectrometer with a diamond ATR accessory. The illumination source used was a mid-IR globar and DTGS detector was used. The beam path was purged with dry, CO₂-scrubbed air. Spectra were obtained at a resolution of 4 cm⁻¹ and 128 scans, with respect to ddH₂O (doubly distilled water) background. Scans were averaged to obtain a good signal-to-noise ratio. Measurements were done on the samples prepared as above and repeated in triplicate. To normalize the FTIR spectra by taking into account the different lengths of the peptides a baseline from the data points at 1480 to 1720 cm⁻¹ was subtracted. The area of the amide I and amide II peaks were calculated using Origin 9.0 and the peak analyser feature. The FTIR spectra were then normalized to the area of the Amide I peak and re-plotted.

2.5.6 Transmission Electron Microscopy (TEM)

Samples were prepared by 40-fold dilution from hydrogels prepared at 40 mg.mL⁻¹. A carbon coated copper (400 mesh) grid from Electron Microscopy Sciences was placed on a 10 μL droplet of the sample for 1 minute. Excess amount of liquid was then drained off onto lint free tissue. The grid was then placed on a 10 μL droplet of ddH₂O for 10 seconds before excess liquid was drained off as before. The grid was then transferred to a 10 μL droplet of a 1 % uranyl acetate solution for 30 seconds; again excess liquid was drained off before the grid was transferred to a 10 μL droplet of ddH₂O for 10 seconds before excess liquid was drained off as before for the very last time. The grid was then stored while it fully dried. Imaging was undertaken on a FEI Tecnai12 BioTwin transmission electron microscope at 100 kV.

2.5.7 Atomic force microscopy (AFM)

Peptide solutions were prepared as above at 1 mg.mL⁻¹ and desired pH. They were then diluted using ddH₂O to a range of concentrations (0.025 mg.mL⁻¹ to 0.5 mg.mL⁻¹). 10 μL of each dilution was dropped onto freshly cleaved mica. After 2 min. excess solution
was removed and the surface was washed once with 1 mL of HPLC grade H₂O. Excess water was then removed once again by wicking using Whatman No.1 filter paper. The samples were allowed to air-dry for one night prior to imaging. Areas of interest were imaged using scan assist mode in air using a Bruker Multimode 8 atomic force microscope with a Nanoscope V controller operating with Nanoscope v8.15 software. Imaging was performed using ScanAsyst Air tips. These silicon nitride probes with Al coating have a nominal radius of curvature of about 2 – 5 nm and a nominal spring constant of 0.4 N.m⁻¹ (Bruker AXS S.A.S, France). Height images with scan sizes of 10 µm × 10 µm were captured at a scan rate of ~0.977 Hz and at a relative humidity of <40%. The instrument was periodically calibrated using a grating with 180 nm deep, 10-mm² depressions. Data was second-order flattened using the Nanoscope Analysis (v1.4) software prior to image export.

2.5.8 Small angle X-ray scattering

SAXS experiments were performed on beamline I22 at the Diamond Light Source (DLS) facility in Didcot, UK. The energy of the beam was 12.4 keV corresponding to an X-ray wavelength of 0.1 nm. Quartz capillaries (1.5 mm outer diameter, 0.01 mm wall thickness) were supplied from the Capillary Tube Supplies Ltd. Samples prepared as above at 2.2 mM, 4.5 mM, 8.9 mM, 13.4 mM (for FK) and 17.6 mM were introduced to capillaries via syringe. Acquisition time was 1 second and the area pixel array detector used to collect SAXS data was a Pilatus P3-2M (from Dectris). The distance between sample and the detector was fixed to 3.47 m, resulting in a momentum transfer vector range of 0.059 (nm⁻¹) < q < 3.067 (nm⁻¹) with q = (4π/λ)sin(θ/2), where θ is the scattering angle and λ the wavelength of incident photons. Calibration of the momentum transfer was performed using silver behenate powder. ddH₂O in a capillary was used as background and subtracted from all measurements, whilst the subtraction mask was created using glassy carbon. Data were reduced using the processing tools in the DawnDiamond software suite. The 2D patterns were integrated using azimuthal integration tool to obtain a 1D scattering patterns. Under these conditions, the coherent absolute intensity scattered by the peptides is as:

\[
I_A(q) = \frac{1}{k}\left[I_N(q) - (1 - C_p)I_S(q) - I_b\right] \tag{5}
\]
where $I_N(q)$ is the normalized intensity scattered by the sample, $I_S(q)$ the normalized intensity scattered by the water, $C_P$ the peptide concentration in g cm$^{-3}$, $I_b$ the background scattering mainly due to the incoherent scattering of the peptides and $K$ the contrast factor expressed as follows:\textsuperscript{39,116}

$$K = \frac{4.76}{m_0^2}(Z_p - \frac{v_pZ_s}{v_s})^2$$

(6)

where $m_p$ is the peptide molecular weight, $Z_p$ and $Z_s$ are the numbers of electrons in the peptide and the water molecules and $v_p$ and $v_s$ their molar volumes, respectively. The background scattering, $I_b$, was estimated using the Porod law which gives the scattered intensity of a two phase system at high q values:\textsuperscript{39,116}

$$I(q) = \frac{K_p}{q^4} + I_b$$

(7)

where $K_p$ is the Porod constant. $I_b$ was estimated by fitting the last 10 data points of the scattering curves using a Porod representation ($q^4I(q)$ vs $q^4$).

2.5.9 Flow-SAXS

Experiments were performed using a Watson-Marlow Sci 323 peristaltic pump. The distance between samples and the detector was fixed to 3.19 m resulting in a momentum transfer vector range of $0.063$ (nm$^{-1}$) $< q < 3.306$ (nm$^{-1}$) with $q = (4\pi\lambda)\sin(\theta/2)$, where $\theta$ is the scattering angle and $\lambda$ the wavelength of incident photons) and all other parameters were the same as above.

2.5.10 Oscillatory rheology

The oscillatory rheology was performed using a Discovery Hybrid 2 (DHR-2) from TA Instruments. A parallel-plate geometry with a 250 μm gap was used. All samples were equilibrated to room temperature prior to characterisation. About 200 μL of sample was placed on the bottom plate using a spatula. Each sample was subject (at $T = 25$ C°) to oscillation frequency mode from 15 Hz to 0.01 Hz at strain 0.2 %, which falls within the linear viscoelastic regime of all samples. All measurements were repeated at least three times. Cyclic stress recovery experiments were performed in two modes:

a) using oscillation time mode with subjecting hydrogels to 0.2 % constant strain for 10 minutes and then increasing the strain to 100 % whilst keeping sample for a further 10 minutes. This process was repeated 6 times in one measurement.
b) using oscillation time mode with subjecting hydrogels to 0.2 % constant strain for 3 minutes and then increasing the strain to 1000 % whilst keeping sample for the further 1 minute to simulate injection. The recovery phase was then recorded using oscillation time mode with 0.2 % constant strain for 120 minutes.

For stress-recovery experiments, ddH$_2$O was trapped in the system to avoid evaporation of sample subject to oscillatory strain and long-time exposures to air. All measurements were repeated at least 3 times to ensure reproducibility. Cyclic rheology was repeated twice on 2 different sample concentrations (26.8 mM and 35.7 mM) to probe the recovery mechanisms as a function of concentration at angular frequency of 6.28 rad.s$^{-1}$.

2.5.11 Rheometer-coupled Shear Induced Polarized Light Imaging (Rheo-SIPLI)

Rheo-SIPLI measurements were performed using a setup described previously.$^{244-246}$ The polarized light imaging device was attached to Anton Paar rheometer (Physica, MCR 301). The Rheo-SIPLI instrument setup consists of a parallel plate rheometer with a bottom transparent plate and a top reflective plate (polished steel mirror), allowing reflected polarized light images to be recorded during shear.$^{246}$ All SIPLI measurements were performed on samples prepared by methods described above. Each sample was firstly placed on the rheometer using a positive displacement pipette. The polarized light images (PLIs) were then taken using a polarizer (P) and an analyser (A) with their polarization planes oriented at 90 degrees to each other (cross polarized) in order to monitor birefringence of the sample. The samples were sheared for 120 seconds at a constant angular speed ($\omega = 0.08$ rad.s$^{-1}$, $\omega = 0.8$ rad.s$^{-1}$ or $\omega = 8.0$ rad.s$^{-1}$) using a parallel-plate geometry with the gap size between the two plates $d=1$ mm. In torsional parallel plate geometry, the shear rate across the sample increases linearly from the centre of an image ($0$ s$^{-1}$) to a maximum at the outer edge of the plate (image), according to the following equation:

$$\dot{\gamma} = \frac{\omega r}{d}$$  \hspace{1cm} (8)

where $\omega$ is angular speed, $d$ is the sample gap and $r$ is radial distance from the centre of the plate. With given angular speeds (0.08, 0.8 and 8 rad.s$^{-1}$), the calculated edge shear rates are: $\dot{\gamma}=1$ s$^{-1}$, $\dot{\gamma}=10$ s$^{-1}$, and $\dot{\gamma}=100$ s$^{-1}$ respectively and these were used in the SIPLI figures in the main text. The PLIs were recorded at a rate 1 images.s$^{-1}$ for 130 seconds followed by
another 60 seconds with a recording rate of 0.2 images s\(^{-1}\) (a relaxation phase of the experiment). All the measurements were performed at room temperature 21 °C (RT).

Figure 27: A schematic representation of the parallel plate shear geometry used for Rheo-SIPLI. The optical setup of a mechano-optical rheometer for simultaneous mechanical rheology and SIPLI measurements. The main optical components of the setup are drawn in solid lines and assigned in bold. Supplementary optical components are drawn in dashed lines and assigned in italic. \(D_o\), \(D_f\), and \(d\) indicate the sheared sample diameter, the top (rotating) disk diameter and the sample thickness (the gap between the shearing surfaces of the rheometer), respectively. Reproduced under Creative Commons Attribution License (CC BY).\(^{244}\)

2.5.12 Ellipsometer measurements

Optical properties of the peptide solutions (<CGC) prepared at 4.6 mM were studied using a Woollam M-2000F focused beam spectroscopic ellipsometer, modified to
allow optical measurements with focusing optics (NA = 0.1) at two angles of incidence: 60° and 65°. At these angles the spot size on the sample was approximately 50 μm × 50 μm. 3 mL of peptide solutions (prepared at pH corresponding to identical charge, as chosen in the main chapter) were placed in cell culture plastic dishes for the ellipsometry characterization taken at wavelengths from 220 nm to 1000 nm.
Chapter 3: Designing hybrid graphene-peptide hydrogels

The work in this chapter was supervised by Prof. Alberto Saiani and Dr Aravind Vijayaraghavan. The outcome from this chapter will be submitted to ACS Nano under the title of:

Fine-tuning molecular interactions between peptide nanofibres and graphene for engineering functional hybrid hydrogels


Author Contributions

W.A.P. prepared rGO/PDADMAC and rGO/PVP samples. M.I prepared all GO, rGO and GO/PDADMAC samples and conceived all graphene-based materials characterisation experiments (Raman spectroscopy, XPS, SEM and ζ-potential). M. Z. performed biocompatibility tests of hMSCs on the hybrid hydrogels. J.M. performed all AFM experiments. J.K.W. prepared all hydrogel samples and conceived all other experiments. J.K.W., A.V. and A.S. co-wrote the manuscript. All authors contributed to analysis of the data and commented the paper.
Abstract

Hybrid hydrogels have attracted wide interest due to their potential in biomedical applications as 3-dimensional (3D) functional scaffolds for engineering tissues. The relationship between molecular structures of nanofibres forming the 3D network and the nano-filler is critical in designing tuneable and functional materials. Here, three β-sheet hydrogel forming peptides: VEVKVEVK (V8), FEFKFEFK (F8) and FEFEFKFE (FE) and five graphene-based materials: graphene oxide (GO), reduced graphene oxide (rGO), three graphene-polymer hybrid flakes: GO with polydiallyldimethylammonium chloride (GO/PDADMAC), rGO with PDADMAC (rGO/PDADMAC) and rGO with polyvinylpyrrolidone (rGO/PVP) were co-assembled to form a selection of hybrid hydrogels. Various interactions between the graphene flakes and the peptides were observed that affected the overall mechanical properties of the hydrogels. Electrostatic interactions and π-π stacking, when phenylalanine residues are present, were shown to play a key role in determining the dispersion of graphene materials in the peptide hydrogels and stiffness of the hybrid materials. In particular, FE with reduced graphene oxide (rGO) and FE with rGO covered with poly(diallyldimethylammonium) chloride (PDADMAC) thin film formed double network-like hybrid hydrogels due to strong formation of peptide nanofibrillar bridges between adjacent rGO flakes. This led to a 3- and 4-fold increase of the storage modulus (G') of these hydrogels in comparison to control. FE hydrogels with homogeneous dispersions of graphene oxide (GO) and reduced graphene oxide (rGO) are further shown to be suitable for 3D culture of human mesenchymal stem cells (hMSCs) with no cytotoxicity. These results focus attention on the importance of understanding interactions between the nano-filler and the nanofibrillar network in forming hybrid hydrogels with tuneable mechanical and biological properties, and demonstrates the possibility of using these materials as 3D cell culture scaffolds for biomedical purposes.

Keywords: Peptide, hydrogels, self-assembly, cells scaffold, graphene materials, stem cells, 3D cell culture
3.1 Introduction

Progress in the biomaterial and tissue engineering fields requires the design of novel functional biomaterials, in particular, functional three dimensional (3D) scaffolds. The materials used must be biocompatible, mechanically tuneable, and offer further attractive physicochemical properties for drug delivery and bio-functionalization. As discussed in the previous chapter, self-assembling β-sheet peptide hydrogels have been shown to hold potential for the construction of such soft materials. Since the primary structure of peptides is easily adjustable, the overall structure can be tuned by controlling peptide concentration, the distribution and sequence of amino acids. Additionally the charge state of the peptide, which is controlled by the pH change, significantly affects the self-assembly process. Control over the self-assembly process can be achieved by a range of stimuli, which include: pH, salt concentration, light, temperature or presence of enzymes and thereby the resulting properties of the hydrogels can be appropriately tuned to specific applications.

The emergence of carbon nanomaterials has provided great opportunity for the use of graphene derivatives (GDs) in biomedical applications. In the recent decade, the incorporation of nano-fillers such as carbon nanotubes or graphene oxide (GO) has emerged as an effective route for the construction of increasingly complex functional materials for a variety of applications ranging from electronics to tissue engineering. Indeed, this approach often resulted in the improvement of the performance or functionality of the hydrogel materials including both physicochemical properties and their biological functionality. One common factor in all these studies is the role of interactions between the biopolymer that forms a hydrogel and the nano-filler. This turns out to be key to the design of the materials with tailored properties. In particular, recently Chen et al. indicated that understanding the way in which graphene interacts with biological molecules is required in order to better design biomaterials.

The incorporation of graphene based nano-fillers further offers potential to tailor mechanical strength of peptide hydrogels, adding binding sites for bio-functionalization with biological molecules, and supplying additional properties such as conductivity for regulating cell behaviours including cell proliferation, differentiation, or protein synthesis, and promotes specific tissue regeneration. Indeed, Kostarelos and Novoselov emphasized that careful design and proper materials characterization are necessary for selecting carbon nanomaterials to achieve a specific biological outcome. Therefore,
understanding the interactions between various graphene-based nano-fillers and the peptide sequences are essential for developing 3D hybrid scaffolds with desirable characteristics.

One of the main aspects when designing materials is to fine-tune the interaction between the nanomaterial and the nano-filler. Wu et al. reported hydrogel construction for a tuneable drug delivery system using covalent hierarchical assembly and chemical cross-linking by means of a photo-linker. However, such complicated schemes often prove difficult in practical use, introduce unnecessary complexity, and require additional time in the material production. Therefore the use of non-covalent approaches is desirable, when the preparation complexity is minimized. Such a method was presented by Sheikholeslam et al. where single-wall carbon nanotubes (SWCNTs) were dispersed in a peptide (FEFEFKFK) solution by simple mixing and ultrasonication. Hydrogels were then simply formed by the addition of the cell culture medium.

\[ \text{V8, F8 and FE (V: valine; F: phenylalanine; K: lysine; E: glutamic acid).} \]

\[ \text{b) Schematic representation of an extended } \beta \text{-sheet fibre.} \]

**Figure 28**: a) Schematic representation of the three designed \( \beta \)-sheet forming peptides: V8, F8 and FE (V: valine; F: phenylalanine; K: lysine; E: glutamic acid). b) Schematic representation of an extended \( \beta \)-sheet fibre.

Due to the simplicity of the structures formed at the molecular level, the relative robustness of \( \beta \)-sheet assemblies and individual graphene flakes, and the ease of functionalization, very stable functional materials with tailored properties can be designed.
In this chapter, a biomaterial system was designed and developed by manipulating the non-covalent interactions at the molecular level between the peptide nanofibrillar network and a variety of graphene based nano-fillers. These interactions were then used to tailor final physicochemical and biological properties of the resulting bulk material for developing functional 3D hydrogel hybrids for biomedical research.

In this chapter the self-assembly of three peptides with 5 graphene materials: graphene oxide (GO), reduced graphene oxide (rGO), three graphene-polymer hybrid flakes: GO with polyallyldimethylammonium chloride (GO/PDADMAC), rGO with PDADMAC (rGO/PDADMAC) and rGO with polyvinylpyrrolidone (rGO/PVP) incorporated into hybrid hydrogels was investigated. The peptides used are: VEVKVEVK (V8), FEFKFEFK (F8) and FEFEFKFE (FE), (V: valine; F: phenylalanine; K: lysine; E: glutamic acid; (Figure 28a). The design of the three chosen β-sheet forming peptides is based on the alternation of hydrophobic and hydrophilic residues originally developed by Zhang et al., similarly to the previous chapter. The alternating amino acids in the peptide design have side chains that strongly interact in water via hydrophobic, complementary hydrogen bonding and electrostatic interactions to promote formation of anti-parallel β-sheet fibrils. Due to the design used, these anti-parallel β-sheet fibrils have hydrophobic residue side chains buried in the fibre core and hydrophilic residue side chains interacting on the outer part of the formed fibre, as shown in Figure 28b.

3.2 Results and Discussion

3.2.1 Peptide hydrogels

Peptide hydrogels were formed from self-assembled β-sheet fibres by sequential pH change at concentrations greater than CGC = 4.5 mM (critical gelation concentration). Initially, the self-assembly process of the three designed peptides: F8, FE and V8 was investigated as a function of pH. All these peptides contain the glutamic acid (E), lysine (K), C- and N-terminus which can be charged depending on the pH (Table 1 in chapter 2) The charge state of all the peptides was calculated theoretically using equation 1 and is presented in Figure 29a as a function of pH. For these calculations, it was assumed that the self-assembly process does not shift pKa values of amino acids. In order to study molecular interactions, the theoretical charge state on the individual peptides was fixed at
+2e\textsuperscript{-} for V8 and F8 (pH 3.5), and -2e\textsuperscript{-} for FE (pH 6). All peptides formed clear, self-supporting hydrogels at pH 3.5 for V8 and F8, and at pH 6 for FE, at concentrations of 8.9, 17.6, 26.8, and 35.7 mM. These pH values were kept constant throughout this study.

**Figure 29**: a) Theoretical charge on each peptide as a function of pH. b) FTIR absorption spectra for the peptide hydrogels prepared at 26.8 mM presenting β-sheet features at 1621 cm\textsuperscript{-1} and 1695 cm\textsuperscript{-1}. SPM images of the self-assembled peptide networks: c) V8, d) F8 and e) FE, prepared at 0.5 mM at chosen pH values.

All formed hydrogels adopted β-sheet structures as revealed by FTIR spectroscopy (**Figure 29b**). Strong absorption peaks at 1621 cm\textsuperscript{-1} and 1695 cm\textsuperscript{-1} were observed, which indicate extended β-sheet secondary structure in peptides.\textsuperscript{234-236} These peptides were then used to formulate self-assembled networks of nanofibres, as revealed by scanning probe microscopy (SPM) (**Figure 29c-e**) at low concentrations of 0.5 mM. Here, slightly different fibrous structures with averagely thicker nanofibres were obtained for V8 compared to the other two peptides. This difference is due to high tendency for fibre association of V8 nanofibres, which was also previously reported for this peptide.\textsuperscript{39}

SAXS was used to investigate the structure of the formed nanofibres in pure hydrogels at a range of lower concentrations (4.5 mM, 8.9 mM and 17.6 mM) (**Figure 30**). Hydrogels of any concentrations larger than 17.6 mM could not be placed in the capillaries
due to their stiffness. There was no scattering obtained for 4.5 mM of V8 sample. Characteristic scattering patterns are presented for the samples in the Figure 30a-c. Peptides at low concentrations, which are close to the CGC (V8 at 8.9 mM, F8 and FE at 4.5 mM) present scattering patterns following $\sim q^{-1}$ behaviour at low q, which is typical for the scattering for long thin rod-like structures. In this concentration regime, the scattering observed is dominated by the form factor of the diluted scattering entities. As explained in the chapter 2, it has been previously shown that for such objects (i.e.: fibres), for $qR_\sigma < 1$ ($R_\sigma$: cross-section radius of gyration) the scattering intensity can be written as:

$$\ln q I(q) \propto -\frac{R_\sigma}{2} q^2$$

(2)

When the observed scattering follows the form described by the above equation, then at low q, a linear behaviour should be obtained in a $\ln[q I_n(q)]$ vs. $q^2$ representation. This is the case and can clearly be seen in the Figure 30d. The cross-section radius of gyration, $R_\sigma$, of the fibres can be estimated from the slope of the linear section. Supposing that the fibres can be modelled by a plain infinitely long cylinder, the $R_\sigma$ is related to the diameter of the fibre, d, through the following equation:

$$R_\sigma = \frac{d^2}{8}$$

(3)

By fittings of the linear regions for all concentrations, average nanofibre diameter sizes can be estimated as: 2.7 ± 0.3 nm, 2.8 ± 0.4 nm and 2.9 ± 0.3 nm, for the V8, F8 and FE respectively. Here, it was assumed that the dominant species in each sample are single fibres. The theoretical length of the oligopeptide in a fully extended $\beta$-sheet conformation is about 2.8 nm with a thickness of 1.5 nm, as previously stated in chapter 2. The obtained values are in good agreement with the theoretical predictions. It is worth noting that for all tested concentrations, nanofibre diameters across three samples were of a very similar order.

The obtained size of V8 did not correspond to the one obtained from SPM technique. The SAXS pattern for V8 at lower concentration 4.5 mM showed no scattering. Indeed, this suggests that for this peptide self-assembly of nanofibres and their respective entanglement within the hydrogel occurs at higher concentrations when comparing to the other two peptides and overall indicates lower tendency for self-assembly. Previously, nanofibres containing phenylalanine were shown to self-assemble at lower concentrations than those containing valine. Not only the self-assembly tendency is different, but also
properties of formed nanofibres vary. It was noted before that β-sheet peptides containing
phenylalanine self-assemble into twisted fibres.\textsuperscript{76, 116} It appears from the SPM
observations, that V8 peptide does not form twisted fibres and instead has higher tendency
for lateral fibre association than the other two peptides described here.

\textbf{Figure 30:} SAXS scattering pattern (log I(q) vs log q) obtained for a) V8, b) F8, c) FE, at
selection of concentrations. d) SAXS scattering pattern at low q plotted in a
\(\ln[q^2(q)]\) vs. \(q^2\) representation. Best fits of curves with linear sections are presented with
the corresponding values of \(R_\sigma\) extracted (see text for more details).

The change in shape of the scattering patterns at high concentrations indicates the
formation of porous structures, and this is clearly visible for the F8 at 17.6 mM (\textbf{Figure}
30b). This suggests that at even higher concentration than 17.6 mM, these peptides will
form porous, mesh-like networks from nanofibres of similar size.

The three peptides at fixed charges were chosen in order to systematically probe
interaction with the selection of graphene derivatives that varied in surface charge and
hydrophobicity. Here, all peptides were kept at similar hydrophobicity (\textbf{Table 1} in chapter
2) which allowed direct comparison between the status of the charge on the peptide (+2e\textsuperscript{-}
for F8 and -2e\textsuperscript{-} for FE) and corresponding π-π stacking interactions (F8 vs V8). Previously,
Nilsson \textit{et al.} showed the model of complementary ionic peptide (FKFKFKFKFK) in which
the aromatic ring from phenylalanine at the first position in sequence interacts with the outer environment. The interaction between such aromatic rings on peptides/proteins and graphene based structures, which are intrinsically built from the aromatic rings arranged in a hexagonal 2D lattice, was already shown in the literature.

3.2.2 Graphene derivatives

\[ \text{Figure 31: Schematic diagram of all used GDs with their surface properties: a) GO, b) rGO, c) GO/PDADMAC, d) rGO/PDADMAC, e) rGO/PVP. Structures of the two used polymers are also depicted in c,d and e. List of } \zeta \text{-potential measurements of the GDs at two different pH values.} \]

The GD nano-fillers used are: graphene oxide (GO) (Figure 31a), reduced graphene oxide (rGO) (Figure 31b), and three graphene-polymer hybrid flakes of GO with polydiallyldimethylammonium chloride (GO/PDADMAC) (Figure 31c), rGO with PDADMAC (rGO/PDADMAC) (Figure 31d) and rGO with polyvinylpyrrolidone (rGO/PVP) (Figure 31e). GO used in this study was prepared by a modified Hummer’s method and was used to form all other graphene-based materials (see materials and methods section). The structures of the two polymers used are also depicted in Figure 31c-e. Thin (nanometre thick) films of the two polymers coating GO and rGO flakes were achieved similarly to previous reports.
**Figure 32:** a) Scanning electron microscopy (SEM) image of spin coated GO flakes on to a Si/SiO\(_2\) wafer with a sub-monolayer coverage and minimal flake overlap. The size distribution is a relatively broad log-normal distribution 16.8 ± 10.1 µm measured from the sizes of 200 flakes from multiple SEM images, measured manually using ImageJ®. b) SPM micrographs of ① graphene oxide (GO), ② reduced graphene oxide (rGO), ③ GO/PDADMAC, ④ rGO/PDADMAC and ⑤ rGO/PVP. Height profiles of individual flakes are shown at places where horizontal lines were drawn on the images. Courtesy of Dr Maria Iliut.

The lateral size distribution of GO and GO based materials was a typically broad log-normal distribution 16.8 ± 10.1 µm (**Figure 32a**). The thicknesses of the flakes vary depending on the chemical nature and surface polymer layers and were probed using SPM (**Figure 32b**). This selection of graphene-based materials was chosen in order to systematically probe interaction with the previously described three peptides that form nanofibres. In particular, 5 chosen graphene-based materials varied in their surface
properties: surface charge, hydrophobicity and available surface for π-π stacking. In order to define surface charges of all graphene-based materials, ζ-potential was measured (at two different pH values corresponding to the pH at which hydrogels were prepared) and is indicated in Figure 31. GO is strongly negatively charged (Figure 31a) and hydrophilic. It is easily dispersed in water and can strongly interact electrostatically with other materials. XPS measurements indicate that almost 30% of the surface of GO is covered with hydrophilic groups (Figure 33a and c), which means that there also remain a large surface area of pure graphene structure available for π-π stacking. Upon reduction, rGO contains fewer (11.8%) of oxygen groups (-OH, -COOH) present on its surface (Figure 33b and d). Therefore its surface charge significantly decreases, yet remains negative. Since then it becomes hydrophobic and not dispersible in water, its exact ζ-potential could not be measured directly. It has high tendency for hydrophobic interactions and almost all surface area available for π-π stacking. In order to modify the surface properties of these two basic materials, the GO and rGO flakes were stabilized in water by the thin coating of two polymers (PDADMAC and PVP).

Figure 33: Wide scan XPS of GO, b) wide scan XPS of rGO c) c1 region of GO d) c1 region of rGO. Courtesy of Dr Maria Iliut.
PDADMAC is a highly cationic polymer that is strongly electrostatically attracted to the surface of GO and rGO. Both of these surfaces were covered in the PDADMAC in order to reverse the surface charge to positive (Figure 31c and 31d). By addition of PDADMAC during reduction process of GO, the rGO/PDADMAC mixture becomes dispersible in water. Non-ionic PVP polymer was also used to fully cover and stabilize rGO flakes during reduction process by purely hydrophobic interactions, similarly to previous reports. The charge of formed rGO/PVP surface is close to neutral at pH 3.5 (Figure 31e).

3.2.3 Study of molecular interactions using scanning probe microscopy (SPM)

Consequently, the self-assembly of the fifteen various combinations of peptides (V8, F8 and FE) and graphene-based materials to form self-supporting hybrid hydrogels was explored. Hybrid materials were prepared as described in the materials and methods section. Whilst all other GDs were easy to mix with the dissolved peptides (see materials and method section), mixture with hydrophobic rGO was difficult to prepare and resulted in phase separation of the two materials. Hence, process for the in situ reduction of GO in the peptide hydrogel was developed (see materials and methods section). Briefly, the reduction was undertaken using cell-friendly ascorbic acid (AA) at 80°C, following the method described by Fernandez-Merino et al.

Reversed phase high-performance liquid chromatography (hplc) (Figure 39) and low mass spectroscopy (ms) (Figure 40-42) confirmed that peptides themselves were stable during this reaction. Raman spectroscopy was also used to monitor the reduction reaction of GO. Figure 34 shows typical D and G bands for GO and rGO. The D band indicates disruption of the symmetrical hexagonal graphitic lattice by either internal structural defects or edged effects and dangling bonds. The G band provides information on the in-plane stretching vibration of symmetric C-C bonds present in the graphene. The \( I_D/I_G \) ratio is typically used to assess the structural changes in graphene based materials. The intensity ratio \( I_D/I_G \) obtained for GO in this work is 0.95 and of rGO 1.0 which suggests higher number of sp2 domains in the rGO due to reduction of oxidative debris. The spectra of rGO embedded in the F8 hydrogel show a shift and a narrowing of the G band which is due to the doping effect caused by the interactions with the hydrogel. Due to the same effect the \( I_D/I_G \) ratio has increased to 1.23. Nevertheless, the in situ reduction of GO inside of a hydrogel does not disrupt the
internal structure of the final rGO significantly when comparing to rGO obtained from reduction in water using same conditions.

Figure 34: Normalized Raman spectra of GO, rGO and rGO inside the F8 hydrogel. Typical D (1350 cm\(^{-1}\)) and G (1580 cm\(^{-1}\)) bands were observed for all three samples.

Initially the interactions between the entangled nanofibrillar networks consisting of β-sheet nanofibres and GDs were probed using SPM. Representative SPM regions of all samples are shown in Figure 35. Samples imaged were prepared at 1:1 weight ratio (0.5 mM peptide to 0.5 mg.mL\(^{-1}\) GD) in order to provide sufficient graphene flakes for imaging their interactions with the peptide nanofibres. It is worth noting that 0.5 mM of peptide is significantly lower than CGC. In the absence of GDs, these nanofibres formed dense networks by the self-assembly process, in which the individual nanofibres interact physically through electrostatic, hydrophobic and π-π interactions with other nanofibres in the network (Figure 29c-e). When mixed with GD, the individual nanofibres (or peptides) also strongly physically interact with the GDs present in the system.

Interactions of V8 peptide (which does not have any aromatic rings present) were firstly probed with the selection of all five GD surfaces (Figure 35a-e). Positively charged V8 nanofibres (+2e\(^{-}\)) were strongly attracted via electrostatic forces to negatively charged surface of GO (ζ=−21.7 mV, Figure 31a) which results in aggregation of peptide nanofibres around the GO (Figure 35a). Fibres were heterogeneously distributed across the micrograph, and places away from GO flakes were free of any peptide nanofibres. Interestingly, peptide nanofibres were observed to form an adhesive bridge between the two GO flakes (white arrow in Figure 35a).
Figure 35: Scanning probe microscopy images of solutions of all hybrid materials. Samples were visualized at 1:1 weight ratio to look at the interaction between formed peptide nanofibres and GDs. White arrows point to particular formed structures, as described in the main text. Courtesy of Dr Jonathan Moffat.
The SPM image of V8 with rGO sample (Figure 35b) shows very short peptide nanofibres adsorbed to the surface of rGO. Here, electrostatic attraction between the two systems is decreased due the presence of fewer oxygen groups (-OH, -COOH, =O) present on surface of rGO in comparison to GO.\(^{264}\) Nevertheless, hydrophobic interactions between the side of the peptide containing valine amino acids and hydrophobic surface of rGO were possible. Indeed, strong adsorption of peptide to the surface of rGO and no presence of nanofibres in the surroundings indicates that hydrophobic interactions played a crucial role here. Overall, the hydrophobic surface of rGO and low affinity of V8 to self-assemble resulted in poor self-assembly of V8 peptide into nanofibres and no further formation of the peptide nanofibrillar network at the low concentration used here (0.5 mM).

The use of strongly cationic PDADMAC polymer that coated the GO and rGO flakes reversed the overall surface charge to highly positive (\(\zeta=38.3\) mV, Figure 31c and \(\zeta=47.3\) mV Figure 31d). V8 peptide in the presence of both GO/PDADMAC and rGO/PDADMAC formed very short nanofibres (Figure 35c and 35d), that appeared thinner than that of control V8 sample (Figure 29c). Here, V8 nanofibres were electrostatically repelled from the highly positive surfaces of GO/PDADMAC and rGO/PDADMAC. Interestingly, for rGO/PDADMAC with higher value of \(\zeta\) –potential, less V8 nanofibres were observed overall (Figure 35d) than for GO/PDADMAC. In particular, it seems that the magnitude of the electrostatic force was sufficiently high and no nanofibres were observed on the surface of the rGO/PDADMAC flakes. Finally, V8 nanofibres in the presence of rGO/PVP seemed very similar to that of the control peptide (Figure 35e and 35c). rGO/PVP has \(\zeta\)-potential close to 0 and no electrostatic interactions could occur between its surface and V8 nanofibres. V8 nanofibres could also interact with the PVP polymer coating on the top of rGO via hydrophobic interactions, but the affinity of these interactions was most likely lower than those with pure rGO surface.

The F8 peptide nanofibres were prepared at the same pH (3.5) to that of V8 and carry the same theoretical charge (+2e\(^{-}\)). The main difference in the structures of V8 and F8 peptides is that F8 peptide contains aromatic rings sticking out of the formed nanofibre, which are prone to undergo short range \(\pi-\pi\) stacking interactions. Similarly to V8, F8 nanofibres aggregated on the surface of GO (Figure 35f) due to strong electrostatic attraction. It appears that the F8 nanofibres can also strongly interact via \(\pi-\pi\) stacking and \(\pi\)-cation interactions through lysine with the surface of GO.\(^{268, 269}\) From SPM image, it can be deduced that surfaces of GO flakes are completely saturated with F8 nanofibres. Once this happens, the overall charge of peptide nanofibres with GO may be close to 0. This may
allow other formed nanofibres to form fibrillar network around the flake and form large number of bridges between the flakes, similarly to V8+GO case (white arrow in Figure 35f). More nanofibres were observed in general for F8 in comparison to V8 due to higher tendency of F8 to self-assemble into nanofibres. The discussed π–π stacking attraction in general should be stronger for rGO. Indeed, F8 nanofibres seem to be very strongly attracted to the rGO flakes (Figure 35g). In particular, attraction forces appear so strong that some of the obtained SPM images show the formation of peptide film rather than aggregation of peptide nanofibres (Figure 43). It was already detected experimentally120, 211 and theoretically270 that the formation of amyloid peptide fibres thought to form in Alzheimer’s diseases can be inhibited by the use of GO, even when both materials hold negative charge. In particular, Li et al. noticed that the self-assembly of Aβ33-42 peptide into nanofibres is shifted towards formation of shorter fibres (550 µM of peptide monomer in 5 µg.mL⁻¹ of GO) or thin film formation with the GO flakes in the environment, at sufficiently high concentration of GO (550 µM of peptide monomer in >20 µg.mL⁻¹ GO).120 This suggests that the observed self-assembly strongly depends on the ratio of concentrations of the peptide to GD.

Both F8 nanofibres and GO/PDADMAC and rGO/PDADMAC flakes hold negative charge and both systems experience electrostatic repulsion. As expected, this resulted in the homogeneously spread nanofibres across the whole SPM images (Figure 35h and 35i). For F8+rGO/PDADMAC dispersion, nice alignment of F8 fibres was achieved due to use of the spin coater for this sample. Previous SPM imaging attempts without using a spin coater to prepare the samples resulted in no sample coverage, possibly due to very strong electrostatic repulsion between the sample and the mica surface. Similarly to V8, F8 in the presence of rGO/PVP flakes (Figure 35j) resulted in very similar fibres to that of the control peptide (Figure 29d).

The general trend in interactions can already be identified. Nevertheless, interactions were further procuring the FE peptide, which holds negative charge (−2e⁻) and contains aromatic rings in its structure. Due to charge difference, FE was prepared at pH 6. At that pH, the ζ-potential value of each GD changed (Figure 31). The most significant change occurred for rGO/PVP, which became negatively charged (ζ=-0.64 → ζ=-21.4, Figure 31e). As expected, when the charge value changed from negative to positive on the peptide (F8 at +2e⁻ → FE at −2e⁻), the nanofibres were electrostatically repelled from the surface of GO and formed homogenous network around the flakes (Figure 35k). Interestingly, negatively charged FE nanofibres were: 1) repulsed via weak electrostatic
interactions and 2) attracted to and adhered via π-π stacking interactions to the surface of rGO (Figure 35l). This combination of the two forces resulted in both a homogeneous network around the rGO flakes but also strongly linked rGO flakes. It is therefore expected that this sample might behave like double network, where the two materials strengthen each other mechanically. Logically, negatively charged FE peptide nanofibres were strongly electrostatically attracted to the surfaces of GO/PDADMAC and rGO/PDADMAC (Figure 35m and 35n). Here, rGO/PDADMAC at pH 6 has significantly higher magnitude of ζ-potential than that of GO/PDADMAC (45.7 mV > 18.5 mV, Figure 31c and 31d). Such strong electrostatic attraction (rGO/PDADMAC) resulted in full adsorption of FE peptide to the surface of rGO/PDADMAC and no nanofibres present around (Figure 35n). On the other hand, with smaller electrostatic repulsion from GO/PDADMAC, FE nanofibres were observed adsorbed to the GO/PDADMAC flake and also formed bridges between the adjacent GO/PDADMAC flakes (white arrow in Figure 35m). FE peptide in the presence of rGO/PVP seems to form small particulates and very few short nanofibres are present on the surface of rGO/PVP flake. Although the FE peptide is weakly electrostatically repulsed from the rGO/PVP at pH 6, it also appears that FE peptide and FE nanofibres were adsorbed to the surface of the rGO/PVP flake (Figure 35o). Hydrophobic interactions between the phenylalanine and PVP polymer seem to be playing a crucial role in this peptide adsorption and appear stronger than electrostatic interactions, since there are no nanofibres present surrounding the rGO/PVP flakes.

Recently, Chen et al. using a combination of molecular dynamics simulations and vibrational spectroscopy found that peptide interactions with graphene depends on the competition between aromatic (planar) and hydrophilic residues present in the peptide. In particular they show that part of one peptide (cecropin P1) stands up on graphene due to an unbalanced distribution of planar and hydrophilic residues, whereas the other one (MSI-78(C1)) lies down on graphene due to an even distribution of phenylalanine residues and hydrophilic residues. Our results confirm the adhesive properties of peptides containing phenylalanine (planar aromatic ring interactions) to the graphene surface, however enhance the examples of interactions between peptides and GDs by taking into account extensive long-range electrostatic interactions. Overall, the self-assembly of peptide nanofibres in the presence of GDs and their subsequent interactions vary significantly depending on the surface properties of GD. Through this preliminary study using SPM it was shown how peptide and GDs flakes interact at the molecular level. These interactions are expected to
significantly affect the physical properties of the formed hybrid hydrogels, when the concentration of the peptide is significantly higher than that used in the SPM experiments.

3.2.4 Interactions in hybrid hydrogels

The bulk hydrogels were prepared at a constant ratio of 26.8 mM of peptide to 0.5 mg mL\(^{-1}\) of GD. Digital pictures of all formed self-supporting hydrogels are presented in the Figure 36a,c,e. Here, the interactions between the two components, and thereby the quality of dispersion, can be assessed qualitatively. Hydrogels with visible aggregation of GD (A) and with homogenous dispersions of GD (H) can be differentiated (Figure 36a,c,e). For GO and GO/PDADMAC samples in the vials, the aggregation was assessed easily due to the slightly transparent brownish colour, whereas for all the other samples which were black, aggregation was only visible on the wall of the vial, where a thin residue layer of the gel remained. Therefore, additional pictures were taken of the individual droplets (20 µL) placed on the thin glass and the overall aggregation state was assessed based on both pictures.

Furthermore, oscillatory rheology was used as a tool to assess quantitatively the viscoelastic properties of formed hybrids. The storage modulus (G') is often used to define stiffness of soft matter materials, such as hydrogels. The intrinsic interactions between objects inside the material contribute to the resulting bulk material mechanical properties. In this case, the stiffness of hybrid hydrogels depends on the form of self-assembled network of nanofibres and on their subsequent interactions with the nano-fillers. The values of G' of all formed materials are presented in Figure 36b,d,f.

Previous SPM experiments gave an insight into the interactions occurring between the nanofibres and GDs. For V8 system with GO and rGO, significant peptide nanofibre and peptide attraction to the flakes was observed. Interestingly, there were many peptide nanofibrillar bridges formed between the adjacent GO flakes with V8 peptide. Indeed, the interactions scale up and can be observed directly in optical pictures of V8 with GO and rGO (Figure 36a). Clearly, the clustering of GO and rGO flakes was observed due to strong electrostatic interactions and formation of peptide bridges that further attract the GO/rGO flakes towards each other. This process leads to the formation of a heterogeneous network, in which large micro-sized clusters would contribute to the mechanical stiffness of the network. Indeed, when G' value of these hydrogels was measured, a clear increase was observed for V8+GO and V8+rGO samples (Figure 36b).
Figure 36: Optical pictures of formed self-supporting hydrogels both in the vials and as droplets (20 µl) for a) V8, c) F8, e) FE. Oscillatory rheology measurements of all formed hydrogels with written $\zeta$-potential values for corresponding graphene derivative (GD) for b) V8, d) F8 and f) FE. Both optical microscopy and oscillatory rheology measurements were performed on concentrations 26.8 mM of peptide and 0.5 mg.mL$^{-1}$ of a given GD. Here, A denotes samples with visible aggregation of GD, whilst H denotes homogeneous dispersions.
On the other hand, for V8 with GO/PDADMAC and rGO/PDADMAC homogenous, unperturbed peptide nanofibrillar networks were previously observed in SPM. Here, GDs did not affect the formed network and clear optical homogenous images were observed in both cases (Figure 36a). Furthermore, no significant difference in G’ between the V8+GO/PDADMAC, V8+rGO/PDADMAC and V8 were observed (Figure 36b). For V8+rGO/PVP, previously small hydrophobic attraction between peptide and PVP polymer was deduced. Again, this may result in formation of smaller aggregates that contributed to a slightly higher value of G’ in comparison to that of the control V8 hydrogel.

To start with, when comparing control F8 to V8 peptide hydrogels, the storage modulus of F8 is significantly higher than that of V8 (4-fold increase). F8 peptide was previously shown by Gao et al. to form very strong networks at that pH due to its symmetric nature and the large quantity of ‘sticky’ junctions between individual nanofibres (via π-π stacking of phenylalanine aromatic rings). The obtained pictures of F8 with all GD hybrids follow the conclusions taken from SPM images (Figure 36c). Indeed, For F8 with GO, rGO and rGO/PVP clear aggregation of micro-sized GD was observed, whereas for F8 with GO/PDADMAC and rGO/PDADMAC, homogenous hydrogels were obtained. However, the obtained rheological properties do not follow the expected pattern. Here, the addition of any GD significantly decreases the G’ value of the hybrid F8 hydrogels (Figure 36d). It is hypothesized that for any GD present, the electrostatic attraction and π-π stacking interactions result in large quantities of F8 peptide and peptide nanofibres being adsorbed to the surface of GO/rGO. Consequently, there is less peptide available for formation of the nanofibrillar network. It is further supposed that interactions between F8 peptide and GO/rGO surfaces are so strong, that at that concentration, polymer coating (PDADMAC/PVP) can be displaced by a film of peptide nanofibres. For example, the strongest repulsion between nanofibres occurs with rGO/PDADMAC (ζ=47.3 mV) and leads to the lowest amount of peptide coating on the rGO flakes under the polymer films. Indeed, this sample had the highest G’ out of all F8 hybrid hydrogels (Figure 36d).

Intuitively, the interactions in FE hydrogels should lead to homogenous dispersions and an increase in G’ value of all hybrid hydrogels. Indeed, all FE hybrid hydrogels were observed to be homogenous (Figure 36e). For FE with GO, due to electrostatic repulsion between nanofibres and GO flakes, the same G’ value was obtained (Figure 36f). When GO was reduced to rGO, as previously described, the mixture of electrostatic repulsion and π-π stacking attraction between FE nanofibres and rGO led to a 3-fold increase in G’ value (Figure 36f). The electrostatic attraction between FE nanofibres and GO/PDADMAC and
rGO/PDADMAC led to the observed increase in the G' values in comparison to control FE hydrogel (*Figure 36f*). Similarly to F8, the FE nanofibres may also experience electrostatic and π-π stacking forces with the GO and rGO flakes beneath the polymer. This combination of electrostatic attraction to PDADMAC, electrostatic repulsion from rGO flakes beneath and π-π stacking attraction led to the highest storage modulus for FE+rGO/PDADMAC (4-fold increase, *Figure 36f*). Similarly, a high increase in G' was obtained for FE+rGO/PVP, suggesting that indeed π-π stacking can occur between aromatic rings present on the FE peptide and the rGO/PVP polymer surface.

These results confirm the importance of long range electrostatic and short range π-π stacking interactions between the peptide nanofibres and GDs nano-fillers in hybrid soft matter materials. As a result, both nano-scale (SPM) and macro-scale (oscillatory rheology) experiments allowed more precise understanding on physical characteristics of the hybrid hydrogels.

### 3.2.5 Peptide-GD hybrids as non-cytotoxic 3D cell scaffolds

**Figure 37**: Day 14 of hMSCs cell live/dead viability in the FE control hydrogels prepared at pH 6 at three different peptide concentrations (a,b,c) and FE hybrid hydrogels prepared at pH 6 with 0.5 mg.mL⁻¹ GO at three different peptide concentrations (d,e,f). Scale bar represents 200 μm. Courtesy of Dr Mi Zhou.
The FE hydrogel and its hybrids with GO and rGO were selected to investigate their bio-compatibility as 3D cell scaffolds. FE-based gels were selected since they were prepared at pH 6, a pH close to physiological pH. Initially, human mesenchymal stem cells (hMSC) were 3D encapsulated in the FE (Figure 37a-c) and FE+GO (Figure 37d-f) hydrogels prepared at three peptide concentrations and 0.5 mg.mL$^{-1}$ GO, cultures being maintained for 14 days. Using live-dead staining, it was clear that cell viability was maintained at >95% over 14-day culture period (green: live cells, red: dead cells) for both control FE hydrogels and hybrid hydrogels with GO. This indicates the non-cytotoxicity of the 3D culture systems for hMSC cells. Hybrid FE hydrogels with rGO were also prepared to check the biocompatibility of the in-situ reduced hydrogels. Again, hMSCs were encapsulated into FE+rGO hybrid hydrogels at two different concentrations (Figure 38). Similarly to FE+GO, the cell viability was maintained >95% for 14 days, indicating that there are differences in cytotoxicity between the GO and rGO on hMSCs.

**Figure 38:** hMSCs live/dead viability in the FE hybrid hydrogels prepared at pH 6 with 0.5 mg.mL$^{-1}$ rGO at two different peptide concentrations: a) 26.8 mM, b) 35.7 mM. Scale bar is 200 µm. Courtesy of Dr Mi Zhou.

Overall, the incorporation of these graphene-based nano-fillers (GO/rGO) into the peptide hydrogels created biocompatible 3D scaffolds for 3D-culture of stem cells with the potential to tune mechanical strengths and to be further bio-functionalized through the adsorption of active biomolecules. Therefore, these hybrid systems provide a versatile platform to fabricate functional scaffolds with applications in the tissue engineering field.
3.3 Conclusions

In summary, three β-sheet nanofibre forming peptides: VEVKVEVK (V8), FEFKFEFK (F8) and FEFEFKFE (FE) were selected in order to probe interactions with a selection of five graphene-based materials. The surface properties of all five graphene materials were varied in terms of the surface charge and hydrophobicity. Clear, self-supporting hydrogels at charge states of ±2 were prepared. Through preliminary studies using SPM, it was shown how peptide and graphene flakes interact at the molecular level and how these interactions affect the physical properties of the formed hybrid hydrogels. In particular, electrostatic interactions and π-π stacking, when phenylalanine residues are present, were shown to play a key role in determining the final properties of the formed materials. When the peptide nanofibres were oppositely charged to the GD, an aggregation of fibres around the flakes and thick nanofibrillar bridges were formed between adjacent flakes that contributed to an increase in G' value of the hybrid hydrogel. When peptide nanofibres were repelled from GD, the formed network had similar G' value to that of the control hydrogel.

In particular, the interplay of miscellaneous molecular interactions between nanofibres and GD resulted in the selection of 3D platforms with tuneable physicochemical and biological properties. To examine this, FE, which forms hydrogels at close to neutral pH was then selected and combined at three different concentrations with either GO or rGO to form 3D hydrogel scaffolds for cell culture. hMSC cells were encapsulated within these hybrid hydrogels and maintained high viability (>95 %) for a culture period of 14 days.

These biocompatible, hybrid hydrogels with tunable mechanical strength are therefore proposed as a versatile platform for culturing cells in 3D. This platform can further be exploited to adjust cell morphology by manipulating cell-nanomaterial interactions and thus holds potential for specific cell functioning and tissue regeneration applications. Further studies related to biomedical and tissue engineering fields, in particular drug delivery and stem cells research, where graphene-based materials can be used as a tool to deliver active bio-molecules will be carried out. Hopefully, the mechanisms discussed here for β-sheet peptides and GDs may enlighten researchers to help design better biomaterials in future.
3.4 Supplementary figures

Figure 39: HPLC chromatographs for all three peptides under reduction with ascorbic acid (AA) and 80°C. All peptides are stable as can be observed by the presence of retention peaks.
Figure 40: Mass spectroscopy trace for F8 peptide (molar mass expected: 1121.28 g mol$^{-1}$) during reduction reaction with AA. All major peaks confirm the stability of the peptide structure during the reaction with AA.
Figure 41: Mass spectroscopy trace for FE peptide (molar mass expected: 1122.22 g.mol\(^{-1}\)) during reduction reaction with AA. All major peaks confirm the stability of the peptide structure during the reaction with AA.
**Figure 42:** Mass spectroscopy trace for V8 peptide (molar mass expected: 929.11 g mol\(^{-1}\)) during reduction reaction with AA. All major peaks confirm the stability of the peptide structure during the reaction with AA.

**Figure 43:** SPM micrographs of F8 with rGO sample. The images show formation of peptide films in the surrounding of rGO flakes. Courtesy of Dr Jonathan Moffat.
3.5 Materials and methods

3.5.1 Peptides

All peptides were purchased from Biomatik (Canada). The peptide purity was confirmed using reverse phase high performance liquid chromatography (HPLC) and mass spectroscopy (MS) with typical purity > 90%.

3.5.2 Graphene oxide (GO) preparation

GO used in this study was prepared by a modified Hummer’s method. Briefly, graphite (10 g, 80 mesh, 94% purity) was first treated with NaNO\textsubscript{3} (9 g, Sigma-Aldrich) and concentrated H\textsubscript{2}SO\textsubscript{4} (338 ml, Sigma-Aldrich) at room temperature (RT) for 3 h to obtain intercalated graphite. The mixture was cooled in an ice bath and 45 g of KMnO\textsubscript{4} (Sigma-Aldrich) were gradually added. After addition of the oxidizing agent, the reaction mixture was stirred at room temperature for 1 week to complete the oxidation. The oxidised graphitic slurry was then diluted with a solution of 5% H\textsubscript{2}SO\textsubscript{4}, followed by the slow addition of 5 g of H\textsubscript{2}O\textsubscript{2} (Sigma-Aldrich) as solution. The resulting graphite oxide was purified by repeated centrifugation and redispersion in DI water until the pH of the supernatant was neutral.

3.5.3 GO:polymer and rGO:polymer preparation

In order to prepare the GO-polymer flakes, first the dry polymer (PDADMAC and PVP) pellets were dissolved into GO dispersion of 2 mg.mL\textsuperscript{-1} concentration, in a ratio of 1:10 GO to polymer by mass of solids and the mixture was homogenised for 5 minutes in a high-shear mixer to ensure uniform ‘stock’ dispersion. The mixture was then washed 3 times with DI water by repeated centrifugation and redispersion to remove any excess polymer that is not adsorbed on the surface of graphene flakes, resulting in a dispersion of GO/polymer hybrid flakes in DI water. In order to prepare the rGO-polymer flakes, the GO to polymer ‘stock’ dispersion containing excess polymer was reduced in-situ at 80 °C for 72h in the presence ascorbic acid as reducing agent at a mass ratio of 1:7 GO to ascorbic acid. Ammonia was then added to produce a basic dispersion (pH ~10). The rGO-polymer was then purified by centrifugation and re-dispersal (3 times) as before to remove the
excess of the polymer resulting in a stable dispersion of rGO-polymer composite flakes in DI water.

3.5.4 Zeta potential (ζ) measurements

The charge of the GDs dispersions was measured at the same concentration of 0.01 mg.mL\(^{-1}\), at pH of 3.5 and 6 using Malvern Zetasizer ESA9800, at room temperature (RT=25 °C). Each solution of GD was titrated using 0.1 M NaOH to the desired pH and concentration prior to measurement. The presented value is an average of three replicates. The measurement can only be done on the dispersible samples. As such, the value of ζ-potential for rGO cannot be defined due to aggregation of highly hydrophobic rGO in water\(^{177}\).

3.5.5 Size distribution of GO dispersions

The size distribution of all GO dispersions were characterised using scanning electron microscopy (SEM), performed on a Zeiss Ultra FEG SEM. Samples were prepared by spin coating GO flakes on to a Si/SiO\(_2\) wafer piece to get sub-monolayer coverage with minimal flake overlap (Figure 32a). The sizes of 200 flakes from multiple SEM images for each suspension were measured manually using ImageJ®, always in the horizontal direction through the centre of the flake in order that shape anisotropies do not skew the distribution results; it is assumed that the drying process does not introduce any orientation anisotropy. The size distribution is a relatively broad log-normal distribution of size, 16.8 ± 10.1 μm, as shown in Figure 32b.

3.5.6 Hydrogel preparation

Peptides were dissolved in 350 or 400 μL of double deionised water (ddH\(_2\)O) using sonication for 30 minutes. Desired GD was then added as a solution. Hydrogels were then prepared by adjusting the pH with NaOH to the desired pH value. The total sample volume was then adjusted by adding ddH\(_2\)O to achieve the desired concentration of the two components. To obtain peptide – rGO hydrogel hybrids, firstly, the GO was added to a dissolved peptide solution. In-situ reduction of graphene oxide then followed by adding ascorbic acid according to this ref.\(^{265}\) This mixture was left for 1 hour at 80°C. The mixture
was then allowed to cool to room temperature and titrated using NaOH to the desired pH. Again, the total sample volume was then adjusted by adding ddH$_2$O to achieve the desired concentration of the two components. All the samples were prepared at room temperature.

3.5.7 Fourier transform infrared (FTIR) spectroscopy

Measurements were performed on a Bruker Vertex 80 FTIR spectrometer with a diamond ATR accessory. The illumination source used was a mid-IR glow bar and DTGS detector was used. The beam path was purged with dry, CO$_2$-scrubbed air. Spectra were obtained using resolution of 4 cm$^{-1}$ and 128 scans, with respect to ddH$_2$O background. Scans were averaged to obtain a good signal-to-noise ratio. Measurements were done on the samples prepared as above and repeated in triplicate. FTIR spectra were normalized by setting β-sheet peak (1621 cm$^{-1}$) as 100% using GraphPad 7.0.

3.5.8 Scanning probe microscopy (SPM)

The SPM measurements of GO, reference rGO and GO/PDADMAC, rGO/PDADMAC and rGO/PVP were performed using a Dimension FastScan microscope (Bruker, USA). Samples were scanned in air, at room temperature using tapping mode with FASTSCAN-A probes (Bruker, USA). The images were acquired at 512x512 pixels resolution over 10x10 um area at a scan rate of 3 Hz. For the substrate preparations, GO, GO/PDADMAC, rGO/PDADMAC, rGO/PVP were diluted from the original dispersions to 0.6 mg.mL$^{-1}$ and deposited with the aid of spin coating on Si/SiO$_2$ substrates. The reference rGO was prepared using a dilute GO dispersion (0.1 mg.mL$^{-1}$) and the same conditions used for the in-situ reduction in the presence of peptides. The rGO substrate was prepared using similar conditions as for other samples. These AFM images were processed using WSxM 5.0 Develop 8.1 software.$^{273}$

Peptide and GD mix solutions were prepared at 0.5 mM and 1:1 ratio of peptide to GD at the desired pH value. All scanning probe microscopy (SPM) measurements on hybrid solutions were carried out using a Cypher S (Oxford Instruments Asylum Research, Santa Barbara, USA). Measurements were acquired in AC mode with AC240TS probes (Olympus Probes, Japan). Images were acquired at a resolution of 512x512 pixels over scan sizes ranging from 20 to 1.5 µm at a scan rate of 1 Hz. Samples were prepared by placing a 10 µL droplet of the suspension on freshly cleaved mica for 2 minutes, followed
by rinsing with water and drying with compressed air. For samples that would not adhere to mica due to surface charge, mica was incubated with a 0.01% solution of poly-L-lysine (Sigma-Aldrich, UK) for 2 minutes and again followed by rinsing with water and drying with compressed air. Areas of the sample were selected to allow visualisation of fibres and the GD flakes. In some cases a continuous film of fibres was created, making it difficult to determine interactions between the fibres and flakes. In these cases, the material was spin coated onto the mica substrate rather than drop-casted.

3.5.9 Reverse phase high performance liquid chromatography (HPLC)

HPLC was used to measure the peptide stability to the reaction of reducing graphene oxide using Ascorbic Acid (Sigma-Aldrich, UK) at 80°C. Samples used for HPLC were prepared at 1 mg.mL⁻¹ at desired pH and diluted by a factor of 30. An analytical scale Phenomenex Jupiter 4µm Proteo column 90Å° (250 x 4.66 mm) was used with a flow rate of 1 mL/min. The column was equilibrated in 90% H₂O / 10% CH₃CN with 0.05% TFA, followed by a 200 µl sample injection. The elution gradient used went from 90% H₂O / 10% CH₃CN to 30 % H₂O / 70 % CH₃CN (all solvents contained 0.05 % of TFA) over 45 min.

3.5.10 Mass spectroscopy (MS)

Peptides solutions were initially prepared at a concentration of 1 mg.ml⁻¹. 2 µl of 30-fold diluted sample was then flow injected into 50%ACN 0.1% formic acid, using an Agilent 1200 series LC system, coupled to an Agilent 6520 QTOF mass spectrometer, ESI positive mode. The data was analysed using Agilent MassHunter software.

3.5.11 Oscillatory rheology

The oscillatory rheology was performed using Discovery Hybrid 2 (DHR-2) instrument from TA Instruments. A parallel-plate geometry with a 250 µm gap was used. All samples were equilibrated to room temperature prior to characterization. About 200 µL of sample was placed on the bottom plate using spatula. Each sample was subject (at RT=25 C°) to oscillation frequency mode from 15 Hz to 0.01 Hz at strain 0.2 %, which
falls within the linear viscoelastic regime of all samples. All measurements were repeated at least three times.

### 3.5.12 Small angle X-ray scattering

SAXS experiments were performed on beamline I22 at the Diamond Light Source (DLS) facility in Didcot, UK. The energy of the beam was 12.4 keV corresponding to an X-ray wavelength of 0.1 nm. Quartz capillaries (1.5 mm outer diameter, 0.01 mm wall thickness) were supplied from the Capillary Tube Supplies Ltd. Samples prepared as above at 4.5 mM, 8.9 mM and 17.6 mM were introduced to capillaries via syringe. Acquisition time was 1 second and the area pixel array detector used to collect SAXS data was a Pilatus P3-2M (from Dectris). The distance between samples and the detector was fixed to 3.47 m, resulting in a momentum transfer vector range of $0.059 \text{ (nm}^{-1}) < q < 3.067 \text{ (nm}^{-1})$ with $q = (4\pi\lambda)\sin(\theta/2)$, where $\theta$ is the scattering angle and $\lambda$ the wavelength of incident photons. Calibration of the momentum transfer was performed using silver behenate powder. ddH$_2$O in a capillary was used as background and subtracted from all measurements, whilst the subtraction mask was created using glassy carbon. Data were reduced using the processing tools in the DawnDiamond software suite. The 2D scattering patterns were integrated using azimuthal integration tool to obtain 1D scattering patterns. Under these conditions, the coherent absolute intensity scattered by the peptides is as:

$$I_A(q) = \frac{1}{K} [I_N(q) - (1 - C_p)I_S(q) - I_b]$$

where $I_N(q)$ is the normalized intensity scattered by the sample, $I_S(q)$ the normalized intensity scattered by the water, $C_p$ the peptide concentration in g cm$^{-3}$, $I_b$ the background scattering mainly due to the incoherent scattering of the peptides and $K$ the contrast factor expressed as follows:\textsuperscript{39, 116}

$$K = \frac{4.76}{m_p^2} (Z_p - \frac{v_p}{v_s} Z_s)^2$$

where $m_p$ is the peptide molecular weight, $Z_p$ and $Z_s$ are the numbers of electrons in the peptide and the water molecules and $v_p$ and $v_s$ their molar volumes, respectively. The background scattering, $I_b$, was estimated using the Porod law which gives the scattered intensity of a two phase system at high q values:\textsuperscript{39, 116}

$$I(q) = \frac{K_p}{q^4} + I_b$$
where $K_p$ is the Porod constant. $I_b$ was estimated by fitting the last 10 data points of the scattering curves using a Porod representation ($q^4I(q)$ vs $q^4$).

### 3.5.13 Cell culture and cytotoxicity measurements

Human mesenchymal stem cells (hMSCs) were grown in $\alpha$-MEM medium supplemented with 10 % v/v FBS, L-ascorbic acid-2 phosphate (10 $\mu$M), 1x Glutamax (Life Invitrogen, UK) and an antibiotic mixture of penicillin (100 units.mL$^{-1}$), streptomycin (100 $\mu$g.mL$^{-1}$) and amphotericin (0.25 $\mu$g.mL$^{-1}$). Cells were regularly sub-cultured at 80 % confluence and cells at < passage 4 were used in the cytotoxicity assessments of the hydrogels.

Hydrogels subjected to the cytotoxicity test included FE, FE+GO, and FE+rGO all of which had a pH of 6 and were prepared at a range of peptide concentrations (17.6 mM, 26.8 mM and 35.7 mM) and graphene-based material concentration of 0.5 mg.mL$^{-1}$. The hydrogels were pre-warmed to 37°C and then the cells were mixed into the hydrogels by gentle pipetting to obtain a final cell concentration of $1.5 \times 10^6$ cells.mL$^{-1}$ of hydrogel. Culture medium surrounding the hydrogels was then replaced 3 times during the initial hour to neutralize the pH, and the cultures were further maintained for 14 days.

Cell viability measurements were performed at day 1 and 14. The cell-containing hydrogels were incubated with a Live/Dead® solution containing calcein AM and ethidium homodimer-1 (Invitrogen, Thermo-Fisher Scientific, UK), and then imaged using an Olympus BX51 fluorescence microscope (emission wavelengths: 515/635 nm, excitation wavelength: 495 nm).

Measurements of cytotoxicity of all other samples (including polymer-coated graphene derivatives) would require a significant amount of time and effort. This forms a future work which will be discussed elsewhere.

### 3.5.14 X-ray photoelectron spectroscopy (XPS)

XPS data were collected on a SPECS custom built system composed of a Phobios 150 hemispherical electron analyser with 1D detector and a microfocus Al K-alpha X-ray source (energy 1486.6 eV). All spectra were collected with pass energy of 20 eV. Combined ultimate resolution as measured from Ag 3d is 0.5 eV with X-ray source and 20 eV pass. The XPS data was processed using CasaXPS software (version 2.3.16 PR 1.6).
All spectra were calibrated to 284.8 eV position corresponding to the C-C peak. The C1s region peak was fitted using Gaussian/Lorentzian shape components (for sp\(^3\) carbon) and asymmetric shape components (for sp\(^2\) carbon) respectively. XPS C1s region was fitted with the synthetic components in the manner which minimizes the total square error fit and corresponds to the literature reports. The samples (GO and rGO) were prepared by drop casting on a cleaned Si/SiO\(_2\) substrate to form a thick film of at least 10 nm (the penetration depth of XPS X-rays is approximately 10 nm).

### 3.5.15 Raman spectroscopy

Raman spectrum of GO and rGO was taken on a Renishaw Raman imaging microscope (inVia) equipped with a Leica microscope and a CCD detector. Raman spectrum was recorded using 532 nm laser line (Cobolt SambaTM continuous wave diode-pumped solid-state laser, 20 mW), and the laser power was kept below 10 µW to avoid thermal degradation of the samples. Raman data were acquired as a line mapping (2 mappings for each sample, 20 points, 1 s integration time, 2 accumulations). The spectra were processed in Wire software (version 4.1). The GO samples were prepared using the same procedure as described above for the XPS. Raman spectra of F8+rGO were measured using Renishaw inVia microscope with excitation radiation by red laser at 632.8 nm and 600 grating. Depth spectra were of hydrogels with 5 μm steps (11 points) in Z direction between each point, 10 s integration time, 5 accumulations.
Chapter 4: Alignment of graphene oxide liquid crystals

The work in this chapter was supervised by Dr Aravind Vijayaraghavan. The outcome from this chapter will be submitted to Nature Materials under the title of:

*Shear banding and macroscopic textures in graphene oxide lyotropic liquid crystals*

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**Author contributions**

All authors conceived and designed the experiments. M.I. synthesised and characterised the GO samples. J.K.W. performed the rheology-based experiments. S.E. developed the mathematical model. O.O.M. contributed to the SIPLI measurements and SAXS interpretation. All authors analysed the data and co-wrote the paper.
Abstract

Graphene oxide (GO) is currently used in a number of processes of technological relevance such as wet spinning, injection moulding or inkjet printing to form graphene fibres, composites and printed conductors. Typically, such processes utilise well-aligned layered GO liquid crystal (LC) structures in aqueous dispersions. Flow and confinement encountered during processing affects the alignment and stability of this phase. In this work, the alignment of GO-LCs of two lateral flake sizes (42.1 ± 29.4 µm and 15.5 ± 7.5 µm) under wide range of rotational shear flow conditions that overlap with the manufacturing processes, defined by angular speeds from 0.08 to 8 rad.s$^{-1}$ (and corresponding maximum shear rates from 0.1 s$^{-1}$ to 100 s$^{-1}$), in real-time, using shear induced polarized light imaging and small angle X-ray scattering, both coupled with in-situ rheometry (Rheo-SIPLI and Rheo-SAXS, respectively) was probed. Under certain conditions, a unique pattern in Rheo-SIPLI: a Maltese cross combined with shear banding was observed. This phenomenon is unique to GO flakes of sufficiently large lateral size. The structure formed is attributed to a helical flow arising from a combination of shear flow and Taylor-vortex type flow, which is reinforced by a mathematical model. The orientations prescribed by this model are consistent with anomalous rheopecty observed in Rheo-SIPLI and an anomalous scattering pattern in Rheo-SAXS. With the current trend towards producing ultra-large GO flakes, evidence that the flow behaviour changes from a Couette flow to a Taylor vortex flow was provided, which would lead to undesired, or alternatively, controllable alignment of GO flakes for a variety of applications.

Keywords

Graphene oxide, liquid crystal, Rheo-SIPLI, Rheo-SAXS, shear banding, Taylor vortex
4.1 Introduction

Graphene oxide (GO) is known to form well aligned layered liquid crystal structures in aqueous dispersions, above certain concentrations and particle sizes.\textsuperscript{149-154} Such a system, known as a lyotropic phase, is typically charge- and sterically stabilised.\textsuperscript{274} It has also been shown that flow and confinement conditions affect the alignment and stability of these phases.\textsuperscript{150, 275, 276} The effect of flow on alignment has been previously reported for a variety of liquid crystals.\textsuperscript{277-281} In particular, Cinader and Burghardt developed a variety of techniques for describing the alignment of liquid crystals in the form of orientation parameters alongside orientation angles and corresponding orientation vectors.\textsuperscript{277} This made way for the rational design of liquid crystal molecules and corresponding methods of their alignment using shear, light or chemical triggers.\textsuperscript{151, 244, 282} It is also known that phenomena such as Taylor vortices and similar instabilities occur during shear flow of liquids.\textsuperscript{283-285} The formation of these instabilities may perturb, improve or induce the alignment of the liquid crystal molecules present in the solvent.\textsuperscript{286}

Over the last decade, increasing attention is paid to processing and potential applications of 2-dimensional (2D) materials. In particular, GO is processed using electrospinning, wet spinning, inkjet printing, injection moulding, etc. to form graphene fibres, composites and printed electronics.\textsuperscript{189, 190, 276} In all of these processes, graphene based dispersions in various media are subject to high shear. Wallace \textit{et al.} discuss the viscoelastic properties of GO dispersions, using as model ultra-large GO sheets with a high aspect ratio (45 000) to study the liquid crystal phase transitions from isotropic to nematic.\textsuperscript{287} They show the typical shear thinning behaviour of GO flakes and link the viscosity to both the liquid crystal phase composition and the concentration. This serves as a motivation to monitor rheological properties of GO dispersions with polarized light imaging to allow easy liquid crystal phase observation \textit{in situ}.

Recently, a method which combines rotational rheology and polarized light imaging has been developed called shear induced polarized light imaging (SIPLI),\textsuperscript{244, 245} which allows optical view of a sample during rheological measurements using various geometries and different states of polarized light. It has since been applied to study the oriented lamellar phase of block copolymers\textsuperscript{246} or shear-induced crystallisation of thermoplastics\textsuperscript{244, 245} and can also be applied to study orientation of liquid crystals during shear flows.\textsuperscript{244} The technique was already described in chapter 2.
In this chapter, the alignment of GO flakes of two lateral sizes (large: GO-L and small: GO-S) in liquid crystalline suspensions under a wide range of shear flow conditions is described. The orientation of GO flakes in-situ and in real-time using shear induced polarized light imaging and small angle X-ray scattering was probed, with both techniques employing an in-situ rheometer (referred to henceforth as Rheo-SIPLI and Rheo-SAXS, respectively). Prior to shear, all GO dispersions showed conventional lyotropic or isotropic phases. Various homogenous and inhomogeneous textures were observed under shear flow. In particular, at high concentration and high shear rate, a unique pattern in SIPLI was observed: a Maltese cross combined with shear banding.

4.2 Results and discussion

In the used Rheo-SIPLI, initially, the light passes through a linear polarizer and becomes polarized, as depicted in the Figure 44a (also Figure 27 from chapter 2). It then goes through a transparent bottom glass plate, interacts with the sample and hits the top reflective plate. The light is then reflected, goes through the sample (again), passes through the bottom transparent plate, a beam splitter (not shown), a linear analyzer crossed at 90 degrees with respect to the polarizer plane and finally reaches a CCD camera (detector). This way, polarized light imaging can be performed on samples, prior to, during and after the application of shear forces. In this case path of the polarized light goes as follows: P → beam splitter → sample → reflective top mirror plate → sample → beam splitter → A → detector. The SIPLI device can be equipped with QWPs in order to record PLIs using circularly-polarized light. This changes the path of the polarized light, which goes as follows: P → QWP → beam splitter → transparent bottom plate → sample → reflective top mirror plate → sample → transparent bottom plate → beam splitter → QWP → A → detector.

Previous reports concerning rheological behaviour and alignment of GO suggested that in a parallel-plate set-up under shear, GO flakes align parallel to the flow direction and perpendicular to both shearing plates as shown schematically in Figure 44b (top view) and Figure 44c (isometric projection). In the SIPLI configuration, the shear flow is in the tangential direction (tracing a circular trajectory around the rheometer axis of rotation) and the resulting orientation of GO flakes should produce a polarized light image illustrated in Figure 44b.
Figure 44: a) A schematic representation of the parallel plate shear geometry used for Rheo-SIPLI. The diameter of the top plate used was kept at 25 mm. The double-sided arrows show plane of polarization of the polarizer (P) and the analyser (A) perpendicular to each other, which are at opposite sides, as previously indicated in Figure 27. The angular speed (ω) indicates the axis and direction of rotation of the top shear plate. b) A Maltese cross; A schematic representation of GO flake alignment directions in the parallel-plate geometry (top view) with a corresponding Maltese Cross which could be observed for this alignment in PLIs, the arrows show plane of polarization of P and A. c) Possible GO flake orientations in the parallel plate shear geometry used for Rheo-SIPLI (parallel, perpendicular, and transverse) schematically depicted with respect to the shearing directions. In the cylindrical coordinate system employed here, x, y and z corresponds to flow, vorticity and shear gradient direction, respectively. We denote the GO flake orientation by the normal to the flake surface.

Light transmission is blocked in regions where the flakes are oriented parallel to either the polarizer or analyzer, and transmitted through the regions where the flakes are at an intermediate angle between the polarizer and analyzer. This pattern comprised of bright and dark regions is typically known as a MC. Such a pattern is commonly observed under
crossed polarisers for birefringent materials in which one of the principal axes of the optical birefringence ellipsoid is always parallel to the radial direction originating from the MC centre, for example in polymer spherulites (in which polymer crystals grow radially from the nucleation centre). Note that in the most general consideration, a sheared suspension of platelets such as clay or graphene can align in three possible orientations or combinations thereof (Figure 44c): transverse, with the flake normal parallel to the flow direction, $x$; perpendicular, with the flake normal parallel to the vorticity direction, $y$; parallel, with the flake normal parallel to the shear gradient direction, $z$. Note that the co-ordinate system employed here is cylindrical, $x$ and $y$ corresponding to tangential and radial directions respectively. If the axis of birefringence ellipsoid of an optically uniaxial material is oriented perpendicular to the platelet normal then under crossed polarizer and analyser, both transverse and perpendicular alignment can produce an MC and parallel alignment produces a dark image (complete extinction) due to the fact that the material is non-birefringent in the $x$-$y$ plane.

Typically, rheological studies of GO involve flakes of lateral sizes no greater than 20 µm and these exhibit shear thinning behaviour. Recently, due to improved manufacturing capabilities, attention has turned to producing ultra-large GO flakes for various applications, such as filtration membranes or printed electronics. At first, large GO flakes (GO-L) with typical log-normal size distribution of 42.1 ± 29.4 µm were considered (Figure 45).
**Figure 45:** The size distribution (log-normal) led to an average size of a) $42.1 \pm 29.4 \, \mu m$ for the GO-L flakes and b) $15.5 \pm 7.5 \, \mu m$ for the GO-S flakes, presented as mean ± standard deviation. These were measured manually using ImageJ® from the sizes of at least 300 flakes from multiple SEM images for each suspension. c,d) Scanning electron microscopy (SEM) image of spin coated GO flakes on to a Si/SiO$_2$ wafer piece with a sub-monolayer coverage with minimal flake overlap. Courtesy of Dr Maria Iliut.

GO used in this study was prepared by a modified Hummer’s method (see materials and methods section for details).$^{179}$ The AFM image shows a monolayer graphene with height of $\sim 1.2$ nm (Figure 46a). The obtained $I_D/I_G=1.01$ (Figure 46b) suggests high number of sp2 domains in the graphene oxide structure.$^{291}$ The XPS measurements show that the oxygen containing groups cover about 27% of GO surface with almost 50% of C-O-C structures present in the GO (Figure 46c-d).
Figure 46: a) AFM micrograph of GO-L showing step height profile of the individual flake. b) Raman spectroscopy of GO showing the typical D and G bands with the obtained ratio of the two peaks: \( I_D/I_G = 1.01 \). c) wide XPS scan of the GO d) \( sp^2 \) carbon region from XPS. Courtesy of Dr Maria Iliut.

Firstly, Rheo-SIPLI was used to study birefringence of aqueous dispersions of GO-L flakes as a function of shear rate, at two different concentrations of GO dispersions (Figure 47). In this experiment, angular speed was increased from an initial value of \( \omega = 0.08 \text{ rad.s}^{-1} \) (Figure 47a and 47b), to \( \omega = 0.8 \text{ rad.s}^{-1} \) (Figure 47c and 47d) and finally \( \omega = 8 \text{ rad.s}^{-1} \) (Figure 47e and 47f). SIPLI images were recorded during a shearing time of 120 s, as well as before and after the shear pulse. In torsional parallel plate geometry, the shear rate across the sample increases linearly from the centre of an image (0 s\(^{-1}\)) to a maximum at the outer edge of the plate according to the equation 4 (previously described in chapter 2): 

\[
\dot{\gamma} = \frac{\omega r}{d} \tag{4}
\]
where $r$ is radial distance from the centre of the plate. With given angular speeds (0.08, 0.8 and 8 rad.s$^{-1}$), the calculated edge shear rates are: $\dot{\gamma} = 1$ s$^{-1}$, $\dot{\gamma} = 10$ s$^{-1}$, and $\dot{\gamma} = 100$ s$^{-1}$ respectively and are given in brackets in the Figure 47. All measurements were performed at a constant gap ($d = 1$ mm).

![SIPLI images of graphene oxide large flake dispersion (GO-L) as a function of concentration (indicated by the top line) and angular speed of rotation (indicated by the left column, shear rates produced at the sample edge are shown in brackets), captured at the end of a 120 s shear pulse. The shearing plates were set at 1 mm gap. SB and MC indicate that shear banding (SB) and/or Maltese cross (MC) is observed. Diameter of the polarized light image (PLI) is 25 mm (corresponding to the diameter of the top plate). The arrows show orientation of the planes of polarization of the polarizer (P) and the analyser (A) perpendicular to each other.](image)

**Figure 47**: SIPLI images of graphene oxide large flake dispersion (GO-L) as a function of concentration (indicated by the top line) and angular speed of rotation (indicated by the left column, shear rates produced at the sample edge are shown in brackets), captured at the end of a 120 s shear pulse. The shearing plates were set at 1 mm gap. SB and MC indicate that shear banding (SB) and/or Maltese cross (MC) is observed. Diameter of the polarized light image (PLI) is 25 mm (corresponding to the diameter of the top plate). The arrows show orientation of the planes of polarization of the polarizer (P) and the analyser (A) perpendicular to each other.

All samples were also imaged under quiescent conditions prior to a shear pulse ($\omega = 0$ rad.s$^{-1}$) and displayed polarized light images of randomly oriented bright and dark regions corresponding to typical lyotropic domains (**Figure 53**).\textsuperscript{152, 153} The PLI pattern changes with angular speed (**Figure 47**). Initially, for 2.2 mg.mL$^{-1}$ GO-L dispersion, the pattern clearly showed an MC at shear rates higher than 0.5 s$^{-1}$ (**Figure 47a**), with a
randomly patterned central region (truncated MC) below this threshold value. Subsequently, the higher the angular speed, the more pronounced the observed Maltese cross (Figure 47c and 47e). Accordingly, the central region radius of the truncated MC became smaller, similarly to what has been observed for polymer melts. This is due to the fact that the position of the central region boundary in the PLIs should, in theory, correspond to the same shear rate value and be independent of the angular speed applied for the rotation. This suggests that the macroscopic alignment and the corresponding truncated MC pattern are associated with a critical shear rate required for the GO flake orientation.

At the higher shear rates striking and very well defined shear banding (B), superimposed on the Maltese cross, was observed for the 2.2 mg.mL$^{-1}$ GO-L dispersion (Figure 47c and 47e). As discussed in detail in below, our observed combination of Maltese cross and shear banding appears very similar to the appearance of well-known banded spherulites under crossed polarizers. Detailed consideration of banded spherulites and related structures has been helpful in interpreting our polarized light images. However, a fundamentally different explanation is required: we have rapidly flowing, liquid samples rather than static crystalline (or liquid crystal) material.

Shear banding in the Couette concentric cylinder geometry is well described, and has recently been reported for aqueous GO suspension (although imaging was not reported and the proposed jamming mechanism produces shear bands parallel to the sheared surface, inconsistent with our findings). There are very few examples of concentric bands in parallel plate geometry such as one used here, with bands only being observed over a narrow range of shear rates and/or only for carefully selected fluids. Bands obtained here are extremely stable (spatially and temporally), very well-defined, form over a very wide range of shear rates and are triggered in water by the addition of a very small amount of GO flake. It is believed to be the first observation and imaging of the combination of Maltese cross in polarized light and shear bands for a sheared liquid.

PLIs of an increased concentration solution of GO dispersion (3 mg.mL$^{-1}$) at the lowest angular speed (Figure 47b), presented a texture of bright and dark regions with crossed polarizer and analyser, corresponding to randomly orientated lyotropic domains. This pattern did not show any Maltese cross. Nevertheless, when the angular speed was increased, the PLIs showed a distinct combination of Maltese cross and shear banding (Figure 47d and 47f). The higher concentration of GO and highest angular speed, produced
the image with the highest contrast (*Figure 47f*). Here, the strong distortion of the Maltese cross in alternating bands was clearly observed.

![Image of optical images](image)

**Figure 48:** Optical images of 3 mg.mL$^{-1}$ GO-L obtained during a shear pulse using angular speed $\omega = 8$ rad.s$^{-1}$ (edge shear rate 100 s$^{-1}$): a) using linearly polarized light represented in polar coordinates as recorded, arrows labelled by $P$ and $A$ define polarization plane orientations of polarizer and analyser, respectively, and b) a corresponding transformed image represented in Cartesian coordinates; c) using circularly-polarized light represented in polar coordinates and d) a corresponding transformed image represented in Cartesian coordinates. Diameter of the PLI is 25 mm (corresponding to the diameter of the top plate). The centre of polar image is transformed to the first vertical line of the Cartesian coordinate picture.

It is worth noting that a 3 mg.mL$^{-1}$ dispersion of GO sheared at $\omega = 0.08$ rad.s$^{-1}$ (*Figure 47b*), produced a texture very similar to the non-sheared case (*Figure 53*). From the experiments, it is clear that upon increasing the angular speed (*Figure 47 a $\rightarrow$ c $\rightarrow$ e and b $\rightarrow$ d $\rightarrow$ f*) the lyotropic phase disappears and a more pronounced, unique texture (SB+MC) in SIPLI can be observed. It is also evident that the most vibrant pattern was observed for 3 mg.mL$^{-1}$. At this concentration, the number of individual GO flakes present in the system was high enough to produce a high contrast image under polarized light.
In order to better visualise and analyse the complex pattern, we perform a polar to Cartesian transformation of the SIPLI images as shown in Figure 48 for the 3 mg.mL$^{-1}$ GO-L sample processed at $\omega = 8$ rad.s$^{-1}$ (edge shear rate 100 s$^{-1}$). Two lines in Figure 48b represent directions (T – tangential and R – radial) along which two different features can be observed: Maltese cross and shear banding, respectively. In order to isolate and observe the shear banding without the Maltese cross (representing isogyres, i.e., the circular dark shadow in the polarized light image, representing the locus of all points that correspond to directions of transmission through the crystal plate in which the state of polarization of the incident rays is unchanged by passage through the plate), Rheo-SIPLI experiments on GO-L were repeated using a circularly polarized light (Figure 48c). In this setup, only isochromatic (isostress) shear band line was observed. The Cartesian polarized light images (CPLI) images (Figure 48d), reveal the fact that the shear bands are continuous and virtually concentric around the axis of rotation. This observation, combined with a closer inspection of the polar and Cartesian representation of the linearly-polarized PLI (Figure 48a and 48b) reveals that in fact the pattern consists of concentric bands, each containing a frustum of the Maltese cross offset in opposite tangential directions in an alternating manner.

In order to represent and analyse the flow and flake alignment leading to these unique patterns, a mathematical model is proposed (Figure 49). This model allowed visualizing the behaviour of GO flakes under variety of flow conditions which allowed finding an alignment that satisfied all experimental conditions (i.e., offset MC in the direction of SBs). Initially, the vector field representing the shear flow in a section of the $y$-$z$ plane was considered (Figure 49a). Using the coordinate system shown in Figure 44c, the flow field resulting from shear $F_s$ can be described as

$$F_s = \left(\frac{r_\omega z}{d}, 0, 0\right)$$  \hspace{1cm} (9) $$

where $d$ is the plate separation i.e., flow in positive $x$ direction with magnitude zero at the stationary bottom plate and magnitude $r_\omega$ at the rotating top plate. Considering only the shear flow, the SIPLI image would look exactly like Figure 44b as discussed above.
Figure 49: Mathematical vector field model was developed to explain and describe the behaviour of GO flakes under a) shear flow, b) vortex flow and c) combined shear and vortex flows. Upon combination of the two fields, the combined field is helical and produces vorticity in x-y plane. d) Top-view flake alignment depicting GO flakes (GO = red lines) aligned with flake normal in the vorticity direction under combined flow. Black and white regions are representing areas where light transmission is blocked (regions where the flakes are oriented parallel to either the polarizer or analyzer) and transmitted (through the regions where the flakes are at an intermediate angle between the polarizer and analyzer), respectively. The presented pattern is the outcome of the mathematical flow fitting and matches well the offset Maltese cross and banding observed in the polarized light image (PLI) from Figure 48. The image on the right shows the formed vortices in z-y plane, between the two plates, taken at a cross section represented as black dashed line on the model image. Courtesy of Dr Stephen Edmondson.
This shear flow field is insufficient to explain the SIPLI images that contain both the cross and banding. We therefore propose that this unexpected and interesting pattern is due to the formation of Taylor-like vortices. Such vortices are more commonly encountered in the Taylor-Couette concentric cylinder geometry but have also been reported in plate-plate geometry. Normally, Taylor vortices occur at high shear rates. Counter-intuitively, the axis of these vortices is known to be tangential i.e., parallel to the flow direction, around the circle.

The observed banded Maltese cross images are superficially similar to those observed under crossed polarizers for banded spherulites due to periodically twisted lamellae, or the ‘spiral stripes’ possible with cholesteric liquid crystals. In these cases, the concentric bands are due to helical changes in alignment, but crucially the helix axis is in the radial direction, rather than in the tangential direction as for the vortex axis in our proposed Taylor-vortex-driven alignment. For 2D flakes such as used here, radial helices would either produce no bands at all (if the flakes are aligned in the perpendicular direction) or produce darker and lighter bands as the flakes alternate between parallel and transverse orientations; no such dark bands are observed. The offset banded Maltese cross pattern is believed to be completely and uniquely explained by the presence of Taylor-like vortices in which the flakes are aligned with the surface normal in the vorticity direction (as for the perpendicular alignment in the non-vortex case).

When Taylor-like tangential vortices are introduced into the structural model Figure 49b, the flow field due to a cylindrical Taylor-like vortex \( F_v \) can be described as

\[
F_v = \left(0, a \left[ z - \frac{d}{2} \right], a \left[ \frac{d}{2} - y \right] \right)
\] (10)

i.e. flow in the \( y-z \) plane increasing in magnitude with distance away from the centre of the vortex at \( \left(0, \frac{d}{2}, \frac{d}{2}\right) \) with the magnitude and sign of \( a \) determining the magnitude and direction of the vortex flow, respectively. It is easy to see that vortex flow itself would not produce the desired pattern in the PLI. Then, assuming the total flow field \( F \) results from a linear combination of shear and vortex flows (Figure 49c), one can write:

\[
F = F_s + F_v = \left( \frac{\tau \omega z}{d}, a \left[ z - \frac{d}{2} \right], a \left[ \frac{d}{2} - y \right] \right)
\] (11)

The vorticity of this flow is given by:

\[
\text{curl} \ F = \nabla \times F = \left(-2a, \frac{\tau \omega}{d}, 0 \right)
\] (12)
i.e., a vector in the x-y plane which is constant throughout the vortex, assuming the vortex is sufficiently small that \( r \omega \) can be considered constant. If one assumes that GO flakes used here are aligned in the perpendicular orientation (with the flake normal parallel to the vorticity direction), flakes orientated perpendicular to the plates and tilted with and angle \( \tan^{-1}\left(\frac{2ad}{\omega}\right) \) to the tangential x direction are predicted, constant throughout the vortex (Figure 49c).

**Figure 50:** a) A coaxial cylinder shearing cell in Rheo-SAXS measurements with two probed locations in the cell depicted: Radial (R) and Tangential (T). The Cartesian coordinate system associated with the dispersion flow was mapped onto the coaxial cylinder cell, where the arrowed black dotted line reproduces a trace of flow and a movement of the Cartesian coordinates. b) Orientation angle of 3 mg.mL\(^{-1}\) GO-L sheared in coaxial cylinder geometry at \( \omega=8 \) rad.s\(^{-1}\) with a 1 mm gap, in the radial direction, as a function of time taken during the shear experiment. Dashed line shows alignment of 90°, which would correspond to the shear-only alignment. 2D SAXS patterns obtained for GO-L at three different time points (3 different frames) are embedded in the graph to show evolution of scattering pattern. Direction of shear is depicted on the 2D patterns by the white arrow.

If one assumes adjacent bands to have values of \( a \) with the same magnitude but opposite sign (i.e., the counter-rotating vortices observed in conventional Taylor-Couette flow), flakes tilted in the opposite direction to the tangential direction are predicted. By inspection (Figure 49d) it can be predicted that the flake alignment described above predicts an offset (tilted) Maltese cross image under crossed polarizers, perfectly matching obtained experimental observations.
In order to confirm the proposed helical vortex flow and explain the orientation of GO flakes and the abrupt changes between bands without forming abrupt domains, an alternative method of analysis, i.e., Rheo-SAXS technique was used in the coaxial cylinder geometry (Figure 50a). The arrangement comprises of two concentric cylinders, a fixed outer cylinder and a rotating inner cylinder providing the two shear surfaces between which fluid is confined. The X-rays impinge on the cylinders laterally, perpendicular to the cylinder walls. The Cartesian co-ordinate system employed in this arrangement is matched to the Cartesian coordinates from the plate-plate setup in SIPLI such that the axes corresponding to the direction of flow and the normal to the shearing planes remains the same in both cases, for the sake of consistent comparison. As a result of the geometry relative to the X-ray direction, the scattering can be measured at two different positions: radial or tangential. This is a significant difference to the orientation between the light rays and the shear flow in SIPLI, where light is always passing through the sample along the z-axis. In Rheo-SAXS, the light passes through the sample along the z-axis in the radial position but along the x-axis in the tangential position. It was already shown that tangential position normally has a 100-fold lower scattering intensity due to the thickness of the cylinder\(^{275}\) and quite often it is difficult to position beam with regards to the coaxial cylinders walls to obtain a reproducible pattern. Hence, 2D scattering patterns were recorded during shear at radial direction and the angle of tilt of the overall anisotropic scattering \((0 \rightarrow 2\pi)\) was calculated using mathematical procedures developed by Cinader and Burghardt:\(^{277}\)

\[
CaB = \sqrt{\langle uu \rangle} = \begin{bmatrix}
\langle \cos^2 \beta \rangle & \langle \sin \beta \cos \beta \rangle \\
\langle \sin \beta \cos \beta \rangle & \langle \sin^2 \beta \rangle
\end{bmatrix}
\]

\[\langle f \beta \rangle = \frac{\int_0^{2\pi} f(\beta) \beta d(\beta)}{\int_0^{2\pi} I(\beta) \beta d(\beta)} \quad (13)
\]

The results of this analysis show (Figure 50) that for GO-L flakes, the angle of tilt throughout 1000 s of shear measurement changed and indicated a tilt in the x-y plane (Figure 50b). On average, the calculated tilt angle was between 75° and 80°. This indicated that angle between the GO flake plane and flow direction was between 10° and 15°, correlating to the offset of the Maltese cross (12.5°) observed in the Rheo-SIPLI images. Throughout the measurement, the angle of tilt was changing. At 20s, at the beginning of the measurements, the angle of tilt was lowest (45°), whereas at 420 s the tilt
angle was highest (90°). This result indicated that GO flakes were undergoing dynamical, abrupt changes, in which they were experiencing forces from combined shear and vortex flow. If there were no vortices (or very weak vortices) present, the angle of tilt would very quickly stabilise to 90° and stay constant throughout the measurement. In this case, the flake alignment in the coaxial cylinder geometry is drawn in Figure 50a and according to the classification presented previously in Figure 44c it corresponds to a perpendicular orientation.

![Figure 51](image)

**Figure 51:** Time sweep rheology of GO-L at 3 mg.mL⁻¹ at ω=8 rad.s⁻¹ (edge shear rate of 100 s⁻¹). During the course of the measurement rheopecty is observed for 3 mg.mL⁻¹ of GO-L. b) Representative SIPLI data showing the evolution of pattern during vortex formation for the first 7 seconds of measurements.

Overall, the observed abrupt changes confirm the formation of Taylor-vortices. Michot and co-workers have already observed dynamic and strong anisotropies in disc-like clay suspensions due to the formation of Taylor instabilities.²⁹⁸⁻³⁰⁰ Unlike case in this chapter, Michot et al. describe alignment of the disc-like particles with the normal in the velocity-gradient direction (discs parallel to plates). Such alignment option cannot match GO system presented here because (a) the SIPLI images would be completely dark and (b) the tilt in the radial Rheo-SAXS would not be observed. The results described here also conflict with Wallace et al.,²⁷⁵ but this will be discussed later.
**Figure 52:** a) PLI of GO-S, as obtained, in polar coordinates. Diameter of the polarized light image (PLI) is 25 mm. P and A define directions of polarizer and analyser. b) Transformed image of GO-S in Cartesian coordinate system. The two line plots T-tangential and R-radial show directions at which two different features can be observed: Maltese cross at T and shear bands at R. c) Quarter Wave Plate SIPLI in Polar coordinates for 3 mg.mL$^{-1}$ GO-S with the shear rate at the edge of 100 s$^{-1}$. Diameter of the polarized light image (PLI) is 25 mm (corresponding to the diameter of the top plate). d) Transformed QWP image of GO-S in Cartesian coordinates. e) Orientation angle of 3 mg.mL$^{-1}$ GO-S sample sheared in coaxial cylinder geometry at $\omega=8$ rad.s$^{-1}$ with a 1 mm gap, in the radial direction, as a function of time taken during the shear experiment. Initial 2D SAXS pattern obtained for GO-S is embedded in the graph. Direction of shear is depicted on the 2D patterns by the white arrow. f) Time sweep rheology of GO-S at 3 mg.mL$^{-1}$ at $\omega=8$ rad.s$^{-1}$ (edge shear rate of 100 s$^{-1}$). During the whole measurement the viscosity was nearly constant and no rheopecty was observed.
Typically, GO dispersions exhibit shear thinning behaviour, where the viscosity decreases with the increase in shear rate. It is predicted that during formation of the instabilities in high shear flow of various dispersions, such as Taylor-vortices formation, additional forces are generated, perpendicular to the direction of flow. Such formation would lead to an increase in the apparent viscosity of the solution and appear as rheopecty, where the viscosity of dispersion increases with time. Such behaviour is typical for lubricants, which solidify or thicken when shaken. Indeed, the expected rheopecty behaviour due to the formation of Taylor-vortices was observed for the whole duration of the GO-L Rheo-SIPLI measurement, with the highest viscosity increase in the first 10 seconds of the measurement (Figure 51a). This rapid change was accompanied by the change in SIPLI pattern, as shown in the Figure 51b. For 3 mg.mL\(^{-1}\) GO-L, it took less than 10 seconds to form fully coherent banding across whole structure, from highest shear rate (100 s\(^{-1}\), edge) to the lowest (0 s\(^{-1}\), centre).

Until recently, most of the studies in this area were performed on GO of lateral size of less than 20 µm. Consequently, the whole investigation on GO with significantly lower size lateral size (GO-S) of relatively broad log-normal distribution: 15.5 ± 7.5 µm (Figure 45) was repeated. Again, prior to starting shear, GO-S exhibited typical lyotropic liquid crystal state (Figure 54). Figure 52a shows the obtained SIPLI image of 3 mg.mL\(^{-1}\) GO-S at an angular speed of ω = 8 rad.s\(^{-1}\). Clearly, the image shows the MC structure. Closer inspection of an image, may suggest shear banding similar to GO-L. However, after an image is transformed using the same procedure as for GO-L, a smeared CPLI of GO-S was obtained (Figure 52b). It was not possible to trace a single band along any vertical (tangential) line (T) on this image. This smeared phase suggested a lack of formation of Taylor-vortices. Figure 52c shows a QWP image of GO-S. Similarly, while at first it may seem that there are clear shear bands present, the transformed Cartesian QWP image (Figure 52d) showed only a smeared phase, with no possibility of tracing a single band across an image (T direction) either. This suggests that only flakes of sufficient lateral size will result in vortex formation and show SB phenomena. To confirm this, the Rheo-SAXS experiments in coaxial geometry were repeated for GO-S (Figure 52e). In particular, very strong anisotropic patterns from GO-S in radial direction indicated that the flakes were aligned along the direction of flow (perpendicular alignment). Mathematical analysis of angle of tilt not only confirmed the perpendicular alignment observed in the 2D scattering patterns (constant 90° angle of tilt throughout the time of measurement), but also showed
no dynamical changes of structure during the whole duration of shear. It is worth noting, that this obtained alignment for GO-S corresponds to the same anomalous patterns obtained by Wallace et al. for the similar flake size distribution of GO to GO-S used in this work. Overall, these results suggest the generation of significantly weaker vortices on the GO-S flakes during shear and point towards the importance of lateral size for the formation of Taylor-vortices driven alignment. As expected, the rheological behaviour of GO-S varied significantly from GO-L. Indeed, as shown in the Figure 52f, no rheopexy was observed for GO-S flakes of the same concentration, sheared in the same conditions as GO-L.

Overall, the obtained results indicated that GO-L aligns due to Taylor-vortices formation, whereas GO-S behaved similar to previous literature reports. To confirm that the observed changes between two samples were not due to large steric changes, zeta potential ($\zeta$) was measured for both flake distribution sizes: $\zeta_{GO-S} = -60.3$ mV and $\zeta_{GO-L} = -53.6$ mV. The difference in charge repulsions between the graphene flakes of different sizes was minimal and was considered not to play a crucial role in the final structure formation.

4.3 Conclusions

In summary, GO flakes of two lateral size distributions: GO-L: 35 ± 1.9 µm and GO-S: 14 ± 1.6 µm were synthesized. Due to high interest of processing of GO with ultra-large lateral sizes, the focus of this work was put on shear-alignment of GO-L flakes. Using Rheo-SIPLI, a combination of polarized light imaging during shear experiments, polarized light images with unique characteristics: shear banding and Maltese cross were obtained. A mathematical model was developed which unravelled the mechanisms of alignment of GO-L flakes at high shear rate (100 s$^{-1}$ on the edge). In particular, relevant images were subjected to a polar to Cartesian coordinate transformation. This allowed visualizing and tracing individual bands across the image. Quarter wave plate (QWP) was utilized in order to remove the Maltese cross (isogyres) from the polarized light images (PLI) and proved that only shear bands remained in the PLIs. This allowed further insights into tracing individual bands in the transformed Cartesian coordinate images. Mathematical modelling revealed that the combination of normal shear-flow and Taylor-vortices flow explains the obtained unique SIPLI patterns for GO-L. Rheo-SAXS on GO-L in coaxial geometry confirmed the tilted
alignment and formation of Taylor-vortices. Rheological measurements were recorded during the Rheo-SIPLI experiments to show viscosity behaviour as a function of time. The observed rheopecty for GO-L flakes at high shear rates (100 s$^{-1}$ at the edge, with angular speed $\omega=8$ rad.s$^{-1}$) confirmed the Taylor-vortices shear alignment theory. Interestingly, combination of Maltese cross and shear banding were observed to form almost immediately after inducing shear (<10 seconds). This indicated the super-sensitivity of GO-L suspensions to shear banding under a very wide range of high-shear conditions. Such super sensitivity could lead to undesirable or, alternatively, controllable textures in GO processed fibres, composites or sensors.

Finally, the GO-S flakes showed similar rheological and structural behaviour to what has been previously reported.$^{149, 154, 275}$ Cartesian SIPLI images of GO-S (with and without QWP) showed a smeared texture, which suggested the lack of alignment. Further Rheo-SAXS and rheological measurements confirmed lack of tilted alignment and lack of rheopecty.

Indeed, here it was shown that the size distribution of GO flakes plays a crucial role in this new Taylor-vortices driven alignment of 2D polymeric structures, using GO as a model. Nowadays, the quality of 2D materials is increasing rapidly, and 2D water-soluble materials of larger lateral sizes are being produced. In parallel, processing capabilities increase and larger shear rates are being used, for example in inkjet printing or wet- and electro-spinning. It is therefore crucial to understand the effect that these shear rates can have on the final structure obtained. For particular applications, one must be careful not to enter this regime of Taylor-vortice driven shear to avoid shear-alignment of 2D structures, which may significantly decrease the quality of the desired device. Likewise, it may be interesting to capture the properties of such shear-aligned structures for particular applications, such as electrical sensors or soft composites for biomedical applications.
4.4 Materials and methods

4.4.1 Graphene oxide preparation

GO was prepared following a modified Hummers method described in detail elsewhere. Briefly, 10 g of natural graphite (30 mesh size, 95% purity) was mixed with 338 ml H$_2$SO$_4$ (98%, Sigma Aldrich) and 9 g of NaNO$_3$ (98%, Alfa Aesar) and left overnight to intercalate. The mixture was then cooled in an ice bath and 45 g of KMnO$_4$ (98%, Alfa Aesar) was added slowly under stirring followed by 6 days of oxidation at room temperature. Afterwards, the brown thick slurry was diluted with 1.1 L of H$_2$SO$_4$ 5% solution followed by the addition of 30 g of H$_2$O$_2$ (30%, Sigma Aldrich) and stirred overnight. The mixture was further diluted with 1 L of solution of H$_2$SO$_4$:H$_2$O$_2$ (3%:0.5%). The graphite oxide purification and exfoliation was achieved by washing the mixture with 3%:0.5% solution of H$_2$SO$_4$:H$_2$O$_2$ and DI water at least 15 times with the aid of centrifugation. The resulting GO was homogeneously dispersed with a vertical mixer.

4.4.2 Graphene oxide flake size reduction

The GO flake size reduction was conducted using a Silverson L5M shear mixer model operating at 9000 rpm for 4 h. During the mixing operation the GO dispersion was kept in an ice bath to prevent it from overheating.

4.4.3 Size distribution of GO dispersions

The size distribution of all GO dispersions were characterised using scanning electron microscopy (SEM), performed on a Zeiss Ultra FEG SEM. Samples were prepared by spin coating GO flakes on to a Si/SiO$_2$ wafer piece to get sub-monolayer coverage with minimal flake overlap. The sizes of 200 flakes from multiple SEM images for each suspension was measured manually using ImageJ®, always in the horizontal direction through the centre of the flake in order that shape anisotropies do not skew the distribution results; it is assumed that the drying process does not introduce any orientation anisotropy.
4.4.4 Atomic Force Microscopy (AFM)

The AFM characterisation of the GO was performed using a Bruker Dimension FastScan probe microscope, operating in tapping mode, with an aluminium coated silicon tip FastScan-A from Bruker. The GO sample was spin coated on a Si/SiO$_2$ (300 nm) substrate using a Laurell spin coater. The silicon substrates were first cut into 1 cm$^2$ pieces, cleaned in acetone, DI water and isopropanol by the aid of sonication and then oven dried. The GO water dispersion of 0.5 mg.mL$^{-1}$ was then deposited using a speed of 3000 rpm and an acceleration of 300 rpm.s$^{-1}$.

4.4.5 X-ray photoelectron spectroscopy (XPS)

XPS data were collected on a SPECS custom built system composed of a Phobios 150 hemispherical electron analyser with 1D detector and a microfocus Al K-alpha X-ray source (energy 1486.6 eV). All spectra were collected with pass energy of 20 eV. Combined ultimate resolution as measured from Ag 3d is 0.5 eV with X-ray source and 20 eV pass. The XPS data was processed using CasaXPS software (version 2.3.16 PR 1.6). All spectra were calibrated to 284.8 eV position corresponding to the C-C peak. The C1s region peak was fitted using Gaussian/Lorentzian shape components (for sp$^3$ carbon) and asymmetric shape components (for sp$^2$ carbon) respectively. XPS C1s region was fitted with the synthetic components in the manner which minimizes the total square error fit and corresponds to the literature reports. The GO sample was prepared by drop casting on a cleaned Si/SiO$_2$ substrate to form a thick film of GO of at least 10 nm (the penetration depth of XPS X-rays is approximately 10 nm).

4.4.6 Raman spectroscopy

Raman spectrum was taken on a Renishaw Raman imaging microscope (inVia) equipped with a Leica microscope and a CCD detector. Raman spectrum was recorded using 532 nm laser line (Cobolt SambaTM continuous wave diode-pumped solid-state laser, 20 mW), and the laser power was kept below 10 µW to avoid thermal degradation of the samples. Raman data were acquired as a line mapping (2 mappings for each sample, 20 points, 1 s integration time, 2 accumulations). The spectra were processed in Wire software.
The GO samples were prepared using the same procedure as described above for the XPS.

### 4.4.7 Zeta potential (ζ) measurements

The charge of the GO dispersions was measured at a concentration of 0.02 mg.mL$^{-1}$ and pH of 6.8 (similar to DI water in which they were dispersed) using a Malvern Zetasizer ESA9800. The measurements were performed at room temperature 21 °C (RT). The presented value is an average of three replicates.

### 4.4.8 Rheometer-coupled Shear Induced Polarized Light Imaging (Rheo-SIPLI)

Rheo-SIPLI measurements were performed using setup described previously.$^{244,246}$ The polarized light imaging device was attached to a rotational stress-controlled rheometer (Physica MCR 301, Anton Paar). Figure 27 already shows the Rheo-SIPLI instrument setup. It consists of a parallel plate rheometer with a bottom transparent plate and a top reflective plate (polished steel mirror), allowing reflected polarized light images to be recorded during shear$^{246}$. Each of the GO samples was firstly placed on the rheometer using positive displacement pipette. The polarized light images (PLIs) were then taken using a polarizer (P) and an analyzer (A) with their polarization planes oriented at 90 degrees to each other (cross polarized) in order to monitor birefringence of the sample. In another setup a circularly polarized light was used for the imaging (CPLI): a Quarter Wave Plate (QWP) was used together with the linear polarizer. The samples were sheared for 120 seconds at a constant angular speed ($\omega = 0.08$ rad.s$^{-1}$, $\omega = 0.8$ rad.s$^{-1}$ or $\omega = 8.0$ rad.s$^{-1}$) using parallel-plate geometry (Figure 44a) with the gap thickness between the two plates $d = 1$ mm. The PLIs for all setups were recorded at a rate 5 images.s$^{-1}$ for 140 seconds followed by another 60 seconds with a recording rate of 0.2 images.s$^{-1}$ (a relaxation phase of the experiment). All the measurements were performed at RT.

### 4.4.9 Rheometer-coupled Small Angle X-ray Scattering (Rheo-SAXS)

Rheo-SAXS experiments were performed on beamline I22 at the Diamond Light Source (DLS) synchrotron (Didcot, UK). The energy of the beam was 12.4 keV corresponding to an X-ray wavelength of 0.1 nm. Acquisition time was set at 20 seconds
and a Pilatus P3-2M area pixel detector (Dectris, Switzerland) was used to collect SAXS data. The distance between samples and the detector was fixed to 3.73 m, resulting in a scattering vector modulus range of $0.0028 \text{ nm}^{-1} < q < 3.6025 \text{ nm}^{-1}$ with $q = (4\pi/\lambda)\sin(\theta)$, where $2\theta$ is the scattering angle and $\lambda$ is the wavelength of incident X-ray photons. Calibration of the scattering patterns was performed using silver behenate powder. Two-dimensional SAXS data were reduced to one-dimensional scattering curves using the Dawn software package available at the station. A rotational stress-controlled rheometer (Physica MCR 501, Anton Paar) equipped with a concentric cylinder shear cell (the cell is made of polished polycarbonate, rotating inner cylinder of radius 24 mm and the sample gap $d = 1$ mm) was mounted on the beamline placed in the beam direction and radial and tangential spots were measured. All the measurements were carried out at RT.

4.5 Supplementary figures

![Figure 53](image.png)

**Figure 53:** Typical polarized light images of lyotropic liquid crystal phase of GO-L prior to shear (at two different concentrations). The vectors show planes of polarization of the polarizer ($P$) and the analyzer ($A$) perpendicular to each other. Diameter of the polarized light image (PLI) is 25 mm (corresponding to the diameter of the top plate).
**Figure 54:** Typical polarized light image of lyotropic liquid crystal phase of GO-S prior to shear. The vectors show planes of polarization of the polarizer (P) and the analyzer (A) perpendicular to each other. Diameter of the polarized light image (PLI) is 25 mm (corresponding to the diameter of the top plate).
Chapter 5: General conclusions and future work
5.1 General summary and conclusions

In the past 20 years, nanomaterials have taken one of the leading roles in technological advancement of humanity. They will soon be used in almost all aspects of our lives. The challenge within this thesis was to exploit the use of simple peptide building blocks via molecular self-assembly for construction of hybrid hydrogel materials with the selection of novel graphene-based materials that had varied surface properties. In particular, the focus of research was put not only on the design of the hybrids, but also into understanding of physical properties of peptide forming hydrogels and graphene oxide liquid crystals (GOLCs). This thesis focused on defining new classes of anisotropy phases of a) peptide nanofibres (chapter 2) b) GOLCs (chapter 4). In chapter 2, four self-assembling nanofibre forming peptides: FEFKFEFK (F8), FKFEFKFK (FK), KFEFKFEFK (KF8) and KFEFKFEFKK (KF8K) were used to demonstrate a new theory of shear thinning, sticky and slippery fibres. In chapter 4, GOLCs were shown to form a novel, previously unseen type of alignment for shear liquids that only occurs for GO flakes of sufficiently large lateral sizes. In these two chapters, links between processing of the materials (shear injection – peptides, wet/electro spinning for graphene) and their relevant properties for given applications was established.

Chapter 3 combines selection of both peptides forming hydrogels and five different graphene based materials for the design of biologically relevant scaffolds. This chapter shows that careful design of molecular components of materials (whether the peptide sequence or surface coverage of GO) impacts on the properties of formed hybrid materials. For tissue regeneration and biomedical applications, materials of great complexity and functionality are still required. It is evident from a literature review that we still lack a scaffold design that has biological, structural and mechanical properties that are compatible with biology. Chapter 3 shows a novel way of forming hybrid materials and fully explains subsequent interactions between the used components.
5.2 Future work

5.2.1 Biomedical applications of hydrogels

There are already many industrially available self-assembling peptide hydrogels, such as one designed originally by Zhang.\textsuperscript{35} Since then, many researchers proposed many designs for a variety of applications. Still, many materials used nowadays lack sufficient functionality, with researchers only recently realizing that copying the extracellular matrix developed by nature is a hard task. The emergence of nanomaterials allows formation of composite and hybrid materials that are increasingly more complex in their function and research is slowly moving towards fully synthetic body parts or scaffolds that can retain many biology and functions at once, whilst having the required physicochemical characteristics. In chapter 3, an attempt to form a selection of such novel materials with controllable physical characteristics was presented. Although physicochemical properties were given, the biological function was only demonstrated on an exemplar peptide with graphene. Many questions are left and are still to be explained. For example, would the defined principles affect β-sheet hairpin peptides? More systematic work also has to be performed, which would look at: 1) the effect of lateral sizes of GD and 2) the effect of the sheet edge interactions of peptide nanofibres (such as F8 or KF8K) with GDs on the final physicochemical and biological properties of formed hybrid hydrogels.

Live/dead pictures of hydrogels indicated small cell morphology changes between the control peptide and hybrid one. An additional staining for cytoplasmic structures should be performed to obtain quantitative results regarding these morphology changes. Such experiments would allow determination of whether the materials can be used as platforms for cell differentiation or not. Another very interesting aspect of graphene encapsulated in hydrogels is the fact that it can interact in many ways with other biomolecules present. Undoubtedly, the known affinity of rGO for π-π stacking and the minimal number of hydrophilic groups on its surface may potentially lead to stronger binding or binding of other bio-molecules than that of GO,\textsuperscript{264} as discussed already in the context of peptide nanofibres. Finally, with scientists wishing to fabricate biomimetic body tissues and organs, it remains a challenge to create hybrid materials, such as peptide hydrogels that are capable of inducing cell differentiation, self-healing, and that are mechanically stable and biologically compatible materials.
5.2.2 Graphene oxide liquid crystals

The whole field of GOLCs is currently in its early years and further research is still required to bring out significant applications that can be practically realized on an industrial scale in the near future. Nevertheless, considering the rapid growth and interest in carbon nanomaterials, it is not surprising that many of the completely novel materials, properties and applications of graphene based LCs will still come. In particular, mixing GOLCs into suitable low molecular weight thermotropic LCs, similarly to what was done by Shakhawan et al. could result in an easy way to obtain controllable, dynamical LCs that can be tuned by electric or magnetic fields. Various designs of doped GOLCs are still yet to come and these may improve electro-optical devices. On the other hand, some of the features of GOLCs, such as lateral size and rheological properties are still understudied, despite their critical importance for the optimal processing of many materials. As shown in the chapter 4, new lyotropic GOLC phase with shear-induced alignment was discovered and occurred clearly for flakes of sufficiently large lateral sizes. As for future work, it would be really interesting to further increase the understanding of these new phases. For example, how it depends on the steric stability of the GOLC flakes. This could be realized in combination with previously mentioned graphene derivatives (GD), such as GO/PDADMAC or GO/PVP, where surface properties significantly vary from conventional GOLCs. The synergetic effect of hybrid peptides + GO solutions and its effect on the alignment of on the hybrid liquid crystals would also be interesting to observe.

Regardless of the many challenges remaining in the field of GOLCs, the research achieved so far is truly inspirational. In order to move forward, scientists from interdisciplinary backgrounds must focus on developing new approaches for formation of hybrid materials, for example, it might be possible to functionalize GOLCs with biological moieties (such as peptides, viruses, antibodies) to form novel phases of GOLCs that can respond to biological molecules or physical factors and become sensors that only require a polarized light source to work. Additionally, dispersing GOLCs in many biopolymers other than peptides, such as DNAs or RNAs might lead to new biologically relevant materials for drug delivery, gene manipulation or the control of cell behaviour.
References


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