

# CHAPTER 2

## Literature Review

## 2.1 Introduction

This chapter deals with a comprehensive literature review which covers the following key aspects: smart polymers (**Section 2.2**); PNIPAAm hydrogels (**Section 2.3**); graft polymerisation methods (**Section 2.4**); and recent advances in cell culture (**Section 2.5**).

## 2.2 Smart polymers

In recent years, “smart polymers” (also known as stimuli responsive polymers, intelligent polymers, or environmental-sensitive polymers) have revolutionised material science. Smart polymers display a unique ability to respond to small changes in an external stimulus by undergoing rapid, dramatic and macroscopic changes in their physio-chemical properties (Galaev and Mattiasson, 1999; Hoffman, 2000; Kumar et al., 2007). The responses are manifested by order of magnitude changes in the material with respect to shape, size, volume, solubility, water content, formation of an intricate self-assembly and/or a sol-to-gel transition (Jeong and Gutowska, 2002). What makes smart polymers so interesting is that their phase transition is reversible and can be easily manipulated (Kumar et al., 2007). The driving forces behind the phase transition varies and could for e.g. include hydrogen bonding, hydrophobic interactions or polymer-polymer interaction, neutralisation of charged groups (by a change in pH or ionic strength), molecular orientation, and/or collapse of polymer system (Galaev and Mattiasson, 1999).

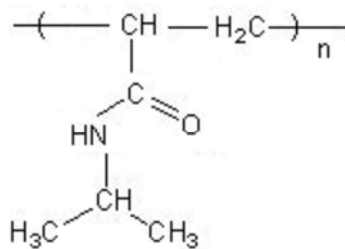
A variety of triggers have been reported in literature and can be classified as follows (Hoffman, 2000; Kumar et al., 2007):

- Physical: temperature, ionic strength, solvents, electromagnetic radiation, electric field, magnetic field;
- Chemical: pH, specific ions, chemical agents; and
- Bio-chemical: enzymes, ligands, and biochemical agents

Thermoresponsive or temperature-sensitive polymers are the most widely studied class of smart polymers since temperature is the sole stimulus for their phase transition and often only modest temperatures are required for a transition to occur (Klouda and Mikos, 2008).

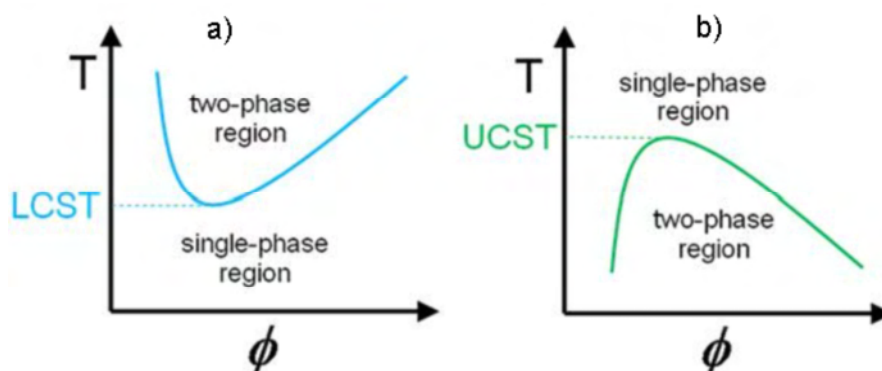
### 2.2.1 Poly(*N*-isopropylacrylamide)

Poly(*N*-isopropylacrylamide) i.e. PNIPAAm is the most popular and well-known of all the thermoresponsive polymers and is the focus of this study. The chemical structure of PNIPAAm is shown in **Figure 2.1**. PNIPAAm is amphiphilic and contains hydrophilic amide groups (-NHCO) and hydrophobic isopropyl groups (-CH(CH<sub>3</sub>)<sub>2</sub>).



**Figure 2.1:** Chemical structure of PNIPAAm.

Thermoresponsive polymers can in general display two types of behaviour i.e. a lower critical solution temperature (LCST) or an upper critical solution temperature (UCST). The LCST and UCST are the respective critical temperature points below and above which the polymer and solvent are completely miscible as shown in **Figure 2.2** (Ward and Georgiou, 2011). Hence a polymer with a LCST becomes insoluble and undergoes phase separation with the solvent as the temperature exceeds its LCST. While for a polymer with a UCST, phase separation occurs when the temperature is below the UCST.



**Figure 2.2:** Schematic showing temperature as a function of polymer volume fraction ( $\phi$ ) for (a) LCST and (b) UCST behaviour of thermoresponsive polymers (Ward and Georgiou, 2011).

## CHAPTER 2: LITERATURE REVIEW

Polymers with a LCST are more widely studied. NIPAAm displays a LCST at ~32 °C which is very useful for biomedical applications since it is close to body temperature (37 °C) (Schild, 1992).

For a polymer in water, three types of interactions are possible, i.e. between polymer molecules, polymer and water molecules, and water molecules (Klouda and Mikos, 2008). For polymers with a LCST, increasing the temperature above the LCST, results in a negative Gibbs free energy ( $\Delta G$ ) according to the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (\text{Eq 2.1})$$

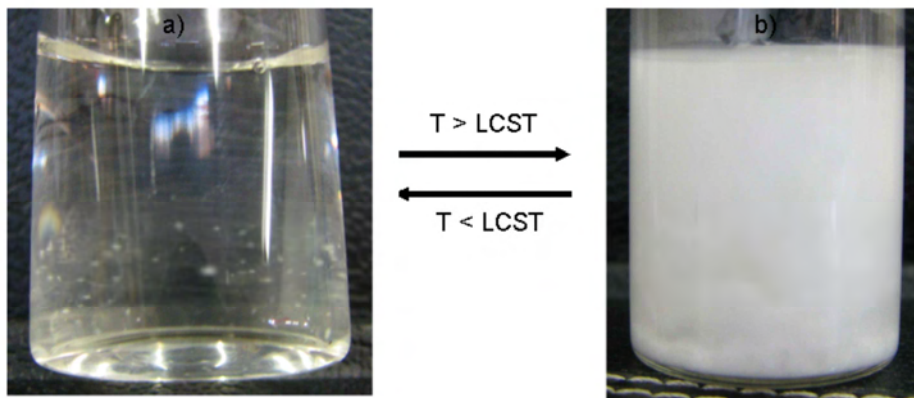
Where  $\Delta H$ , and  $\Delta S$  refer to the change in enthalpy, and entropy respectively.

The main driving force for the negative  $\Delta G$  when the temperature exceeds the LCST, is the increase in entropy of the system due to water-water interactions when the polymer is not in solution (Klouda and Mikos, 2008; Ward and Georgiou, 2011). This favours polymer-polymer interactions while making polymer-water associations unfavourable. The phenomenon above the LCST is also known as the hydrophobic effect (Klouda and Mikos, 2008; Ward and Georgiou, 2011). However when the temperature is reduced to below the LCST, the exothermic  $\Delta H$  enthalpic effects dominates due to hydrogen bonding between the hydrophilic groups in the polymer and water molecules which is the initial driving force for dissolution, swelling or expansion of the polymer chains (Schild, 1992). Likewise UCST is also an enthalpic driven effect (Ward and Georgiou, 2011).

PNIPAAm can exist in three categories based on its physical form and each displays a typical response at the LCST. This includes PNIPAAm in solution; PNIPAAm hydrogels; or PNIPAAm layers on a solid surface (Hoffman, 2000; Jeong and Gutowska, 2002; Kumar et al., 2007). For the different categories, the following changes are expected at the LCST:

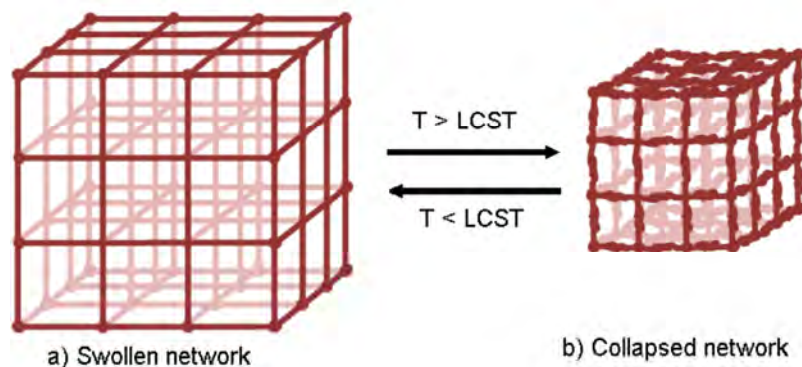
- **PNIPAAm in solution** – These polymers display linear mobile chains in solution which are extended in the coil configuration below the LCST. Upon heating above the LCST, the linear polymer undergoes a reversible conformational change from disordered random coils to a compact globular form. This is often associated with a change in turbidity of the solution and precipitation or gelation as shown in **Figure 2.3**.

## CHAPTER 2: LITERATURE REVIEW



**Figure 2.3:** Photo showing phase transition of PNIPAAm solution (a) at 23 °C ( $T < LCST$ ) and (b) at 40 °C ( $T > LCST$ ).

- PNIPAAm hydrogels** - Thermoresponsive hydrogels are generally highly swollen cross-linked polymer networks below the LCST, while the cross-linked polymer chains abruptly collapse and the polymer phase separates above the LCST. This manifests in shrinking of the hydrogel, and expulsion of water as shown in **Figure 2.4.**, and the formation of a white precipitate.



**Figure 2.4:** Schematic showing effect of temperature on a cross-linked PNIPAAm hydrogel which changes from the (a) swollen network to (b) collapsed network when the temperature is raised above the LCST (Ward and Georgiou, 2011).

- PNIPAAm layer on a solid surface** - This refers to a solid surface modified by a PNIPAAm layer either by physical adsorption of a smart polymer onto the surface, or by covalent bonding i.e. grafting. Below the LCST, the PNIPAAm chains extend away from the solid surface, while above the LCST the polymer chains collapse on the surface as a result of the change in hydrophilicity/hydrophobicity at the solid-liquid interface (**Figure 2.5**).



**Figure 2.5:** Image showing the phase transition of PNIPAAm attached to a surface at temperatures below and above the LCST (Cooperstein and Canavan, 2010).

A lot of research has been reported with respect to PNIPAAm hydrogels and PNIPAAm grafted surfaces and these will be dealt with in **Sections 2.3 and 2.4** respectively.

## 2.3 PNIPAAm hydrogels

A hydrogel is a cross-linked polymer network capable of absorbing and retaining large quantities of water in its porous structure (Hennink and van Nostrum, 2002). The water holding capacity of the hydrogels arise mainly due to the presence of hydrophilic groups, i.e. amides (CONH), carboxyl (-COOH) and hydroxyl (-OH), in the polymer chains capable of forming hydrogen bonds with water molecules (Pal et al., 2009). The amount of water in a hydrogel can vary from 10% to as much as thousand times the weight of the xerogel (Pal et al., 2009). A xerogel is defined as the dried polymer network. One of the key elements of hydrogels is that it contain cross-links in its structure which enables penetration of water into the polymer network enabling the material to swell without dissolution (Milichovsky, 2010). The cross-linked structure provides hydrogels with a 3D structure.

Hydrogels can be classified as either permanent or physical hydrogels depending on the crosslinking. The former involves covalent bonds between the polymeric chains e.g. via the use of a chemical crosslinker, while the latter involves physical interactions (such as hydrogen bonding, ionic interaction, and/or interpenetrating networks) (Pal et al., 2009).

Hydrogels are particularly attractive for biological applications due to their soft consistency, and high water content which is similar to native tissue (Geever et al., 2007). Additionally hydrogels generally display good biocompatibility due to their

## CHAPTER 2: LITERATURE REVIEW

hydrophilic surface which typically display a low interfacial free energy in body fluids, and is non-adhesive to proteins and cells (Hennink and van Nostrum, 2002).

The most common reported synthesis methods for PNIPAAm hydrogels include chemical crosslinking by the use of free-radical polymerisation (Grinberg et al., 2000; Ortega et al., 2007; Pekcan and Kara, 2003; Zhang et al., 2005; Zhang et al., 2003a; Zhang et al., 2003b; Zhang et al., 2002b), photopolymerisation (Geever et al., 2006; Geever et al., 2007), plasma-radiation, and gamma-irradiation (Kishi et al., 1997; Ortega et al., 2007). Recently controlled polymerisation techniques such as atom transfer radical polymerisation (ATRP) and radical addition fragmentation transfer polymerisation (RAFT) have been used to synthesis PNIPAAm hydrogels (Liu et al., 2006).

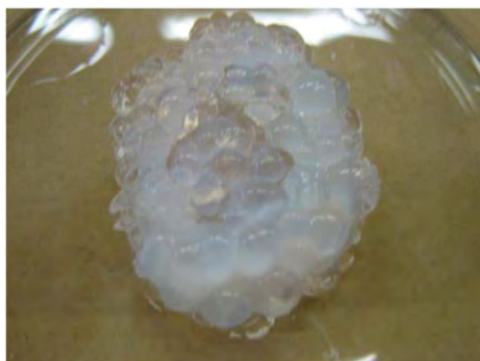
Extensive work has been conducted with respect to modifying the LCST of PNIPAAm hydrogels by copolymerising NIPAAm with either hydrophilic or hydrophobic monomers as shown in **Table 2.1**. It is well-known that copolymerisation of *N*-isopropylacrylamide (NIPAAm) with hydrophilic monomers increases the LCST, due to stronger water-polymer interactions, while the use of hydrophobic co-monomers decrease the LCST due to increase in polymer-polymer interactions. NIPAAm has been polymerised with a number of other monomers including acrylamide; poly(ethylene glycol) methacrylate; poly (1-vinyl-2-pyrrolidone) amongst others (Geever et al., 2007). Attempts have also been made to render PNIPAAm hydrogels with dual stimuli response.

Despite the favourable properties of PNIPAAm, these smart polymeric hydrogels display two major limitations, i.e. poor mechanical properties and slow response time to temperature changes (Zhang et al., 2008). The slow response rate to temperature is believed to be due to the formation of a dense skin layer which forms as a result of the strong hydrophobic interactions existing among the isopropyl groups in the PNIPAAm chains which retards the outward diffusion of water molecules during the hydrogel-collapse process at temperatures above the LCST (**Figure 2.6**). Additionally, the swelling rate of the hydrogel at temperatures below LCST is even slower (Zhang et al., 2008).

## CHAPTER 2: LITERATURE REVIEW

**Table 2.1:** LCST of various PNIPAAm copolymers (Liu et al., 2009).

| Abbreviation            | Co-monomer   | LCST / °C            |
|-------------------------|--|----------------------|
| P(NIPAAm-co-PAC)        | 4-Pentenoic acid                                     | 19.2–36.5            |
| P(NIPAAm-co-MAAm)       | Methacrylamide                                       | 32.4–43.2            |
| P(NIPAAm-co-PAA)        | Propylacrylic acid                                   | Insoluble to soluble |
| P(NIPAAm-co-VPBA)       | Vinylphenylboronic acid                              | 20–40                |
| P(NIPAAm-co-AAm)        | Acrylamide   | 34.7–100             |
| P(NIPAAm-co-NIPMAm)     | N-Isopropylmethacrylamide                            | 34–45.6              |
| P(NIPAAm-co-HEMAm)      | 2-Hydroxyethylmethacrylamide                         | 21.4–30.3            |
| P(NIPAAm-co-VL)         | Vinyl laurate  | <16                  |
| P(NIPAAm-co-VP)         | N-Vinyl-2-pyrrolidone                                | 32.2–39.6            |
| P(NIPAAm-co-NHMA)       | N-Hydroxymethylacrylamide                            | 32–80                |
| P(NIPAAm-co-NVA)        | N-Vinylacetamide                                     | 30–60                |
| P(NIPAAm-co-MVA)        | N-Methyl-N-vinylacetamide                            | 33.2–39.1            |
| P(NIPAAm-co-ACMP)       | 4-Acryloylmorpholine                                 | 31.1–35.3            |
| P(NIPAAm-co-DMAm)       | N,N-Dimethylacrylamide                               | 32–72                |
| P(NIPAAm-co-(Ac-AAs))   | N-Acryloyl amino-alkylacides                         | 23–36                |
| P(NIPAAm-co-HIPAm)      | 2-Hydroxyisopropylacrylamide                         | 32–80                |
| P(NIPAAm-co-MAH)        | 2-Methacryloamidohistidine                           | 31–35                |
| P(NIPAAm-co-PEGMA)      | Poly(ethylene glycol) methacrylate                   | 34–39.5              |
| P(NIPAAm-co-BCAA)       | Benzo-15-crown-5-acrylamide                          | 22–32                |
| P(NIPAAm-co-DMA-co-BMA) | 2-(Diethylamino)ethyl methacrylate butylmethacrylate | 20.3–28.4            |


**Figure 2.6:** Image showing dense skin formation on bulk PNIPAAm hydrogels after de-swelling at 60 °C for one hour (Geever et al., 2007).

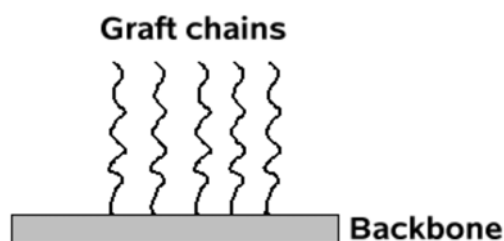


Due to these limitations the use of PNIPAAm hydrogels has been limited in some applications such as cell culture, molecular on-off switches, artificial organs, drug encapsulation, and actuators (Zhang et al., 2008). Improving the response rate of PNIPAAm hydrogels has been a major research focus for many groups (Woodward et al., 2003; Xue et al., 2002; Zhang et al., 2002a; Zhang et al., 2005; Zhang et al., 2003a).

To improve the properties of PNIPAAm hydrogels, various strategies have been employed which include cross-linking; synthesis of a heterogeneous hydrogel structure (e.g. using mixed solvents); the use of porogens (e.g. polyethylene glycol); the use of hydrophilic co-polymers; and cold polymerisation (to create a porous structure) amongst others (Zhang et al., 2008). In this study we investigate the effect of crosslinking and co-polymerisation using mixed solvent systems, on the physical properties of PNIPAAm hydrogels (**Chapter 4**).

## 2.4 Graft polymerisation methods

A number of strategies have been reported regarding grafting of PNIPAAm onto polymer substrates. Grafting or graft polymerisation refers to the chemical attachment of a monomer or growing macroradical via a covalent bond onto a solid polymer backbone as shown in **Figure 2.7**.



**Figure 2.7:** Schematic representation of a graft polymer on a polymer backbone.

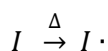
Grafting involves the generation of free radicals, and the breaking and creation of covalent bonds. Graft polymerisation is often preferred over physical adsorption since covalent attachment of PNIPAAm graft chains onto a backbone polymer assures their long-term stability as opposed to a physically attached graft layer where leaching of the adsorbed layer after continued use may occur, which may pose a problem if the scaffold will be re-used. Graft polymerisation offers the advantage of

## CHAPTER 2: LITERATURE REVIEW

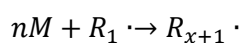
enabling materials to be developed with tailored surface properties while maintaining their bulk properties.

Typically graft polymerisation can occur via two mechanisms i.e. either free-radical or ionic (Desai and Singh, 2004). Free-radical polymerisation is commonly used to polymerise monomers into polymers, and this method of polymerisation typically proceeds via three sequential steps i.e. initiation, propagation and termination (**Scheme 2.1**). Initiation involves the use of an initiator species which can be a chemical initiator, or a high-energy source, capable of forming free radicals on the monomer. After the initiation reaction, chain propagation occurs which involves addition of monomer units to the initiated monomer species, until the growing monomer radicals terminate. Termination can occur by combination, disproportionation or by chain transfer reactions.

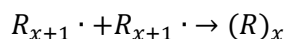
Initiation:



Propogation:



Termination:



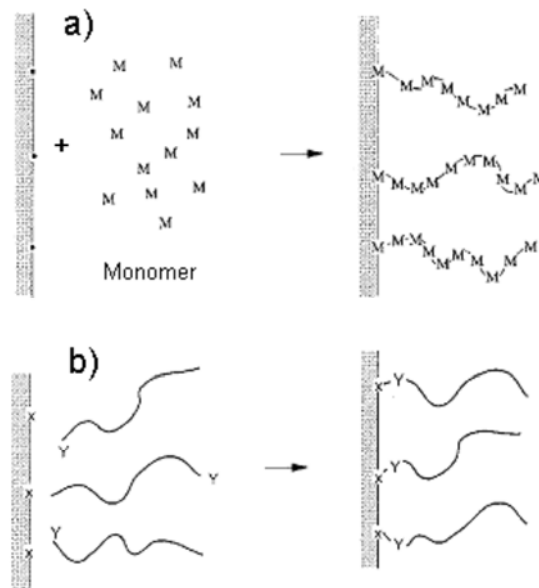
**Scheme 2.1:** Mechanism showing typical steps involved in polymerisation, where I, M, and R refers to the initiator, monomer, and propagating radical respectively and n and x refer to number of units of monomer and propagating radical respectively.

In the case of graft polymerisation, the graft polymer as well as the homopolymer can form in solution, and both processes compete for monomer. For formation of the graft polymer, grafting can occur either “from” the polymer backbone or “to” the polymer backbone as classified below (**Figure 2.8**):

- **Grafting from:** Initiation occurs on the polymer backbone at an active site which forms a free-radical on the polymer backbone. The polymer radical is then transferred onto the monomer thereby propagating the growing monomer radical from the backbone polymer (Huang and Sundberg, 1995).

## CHAPTER 2: LITERATURE REVIEW

- **Grafting to:** The monomer is propagated into a growing monomer radical or polymer chain with reactive end groups which can then covalently coupled onto the polymer backbone at a reactive site (Kato et al., 2003).



**Figure 2.8:** Schematic illustrating the (a) “grafting from” and (b) “grafting to” concepts (Uyama et al., 1998).

Formation of a free radical on the polymer backbone typically occurs either by hydrogen abstraction, breaking of weak covalent bonds (such as O-O), or breaking of unsaturated bonds, which creates a graft site on the polymer backbone. Free-radical induced grafting techniques which have been applied for attachment of PNIPAAm onto surfaces include plasma (Cheng et al., 2005; Kim et al., 2002; Liang et al., 2000; Wang and McCord, 2007), photochemical (Curti et al., 2005; Liang et al., 1999), electron-beam (Akiyama et al., 2004; Bucio et al., 2005; Okano et al., 1995), gamma radiation (Bucio et al., 2006; Contreras-Garcia et al., 2008; Mele'ndez-Ortiz et al., 2009; Ramirez-Fuentes et al., 2007), and chemical (Curti et al., 2002; Gupta and Khandekar, 2003). In recent years living radical polymerisation has also been employed to develop PNIPAAm graft chains with controlled chain lengths and molecular weights (Desai et al., 2003; Wan et al., 2009).

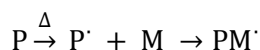
### 2.4.1 Radiation-induced graft polymerisation

Radiation-induced graft polymerisation techniques can be classified as high-energy radiation or ionisation radiation (electron beam, X-rays and  $\gamma$ -rays), mid-energy

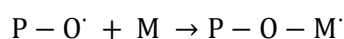
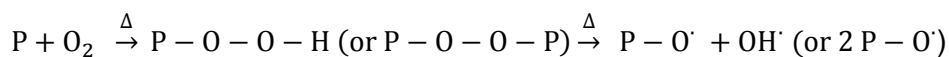
## CHAPTER 2: LITERATURE REVIEW

(ultraviolet-visible/photoradiation and plasma radiation) and low energy radiation (infrared, microwave and ultrasonic radiation) (Desai and Singh, 2004). Radiation of polymers with high energy can cause cleavage of bonds since the energies are often larger than that of covalent bonds and hence free radicals can form directly on the polymer backbone (Bhattacharya and Misra, 2004). Grafting can proceed in three ways, i.e. (a) pre-irradiation (b) peroxidation and (c) mutual irradiation technique (Bhattacharya and Misra, 2004) as shown in **Scheme 2.2**.

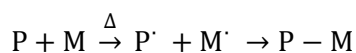
Pre-irradiation:



Peroxidation:



Mutual irradiation:



**Scheme 2.2:** Mechanism for high-energy graft polymerisation induced by radiation (Bhattacharya and Misra, 2004).

The pre-irradiation method involves irradiation of the polymer backbone to form free radicals which is then reacted with the monomer to induce graft polymerisation (Bhattacharya and Misra, 2004). In the peroxidation method, which sometimes is also referred to as pre-irradiation by some authors, the polymer is irradiated in the presence of air or oxygen to form hydroperoxide or peroxide groups which can be activated by heat to induce graft polymerisation in the presence of the monomer (Bucio et al., 2006; Contreras-Garcia et al., 2008; Ramirez-Fuentes et al., 2007), while in the mutual method both monomer and polymer are radiated simultaneously. The advantage of the pre-irradiation and per-oxidation methods is that since the monomer is not irradiated, grafting is typically free from homopolymerisation. The commonly used radiation-induced graft polymerisation methods, which have been used for development of PNIPAAm grafted polymeric scaffolds, are based on gamma-radiation, electron-beam, plasma radiation, and photo-irradiation, amongst others.

### 2.4.1.1 Gamma radiation and electron-beam radiation

Gamma and electron-beam radiation are commonly used industrial radiation processes which are based on high-energy electrons (0.1–10 MeV) and cobalt-60 ( $\text{Co}_{60}$ ) (~1.25 MeV) respectively (Desai and Singh, 2004). During the process, electrons are displaced from atoms and molecules producing ions. The advantage of the high-energy radiation techniques is that a chemical initiator is not required, and graft polymerisation can be carried out without any toxic chemicals. Gamma radiation is also known to have a higher depth of penetration (Clough, 2001). A number of studies have been conducted regarding grafting of PNIPAAm onto polymer backbones using gamma-induced radiation (Bucio et al., 2006; Contreras-Garcia et al., 2008; Meléndez-Ortiz et al., 2009; Ramirez-Fuentes et al., 2007). Okano *et al* first prepared PNIPAAm grafted tissue culture trays by coating the tissue culture tray with a NIPAAm-solvent solution, and then irradiated the surface with electron beam radiation, and recently PNIPAAm grafted cell culture trays called RepCell which are prepared by electron beam irradiation is commercially available (Hutmacher, 2005; Okano et al., 1995).

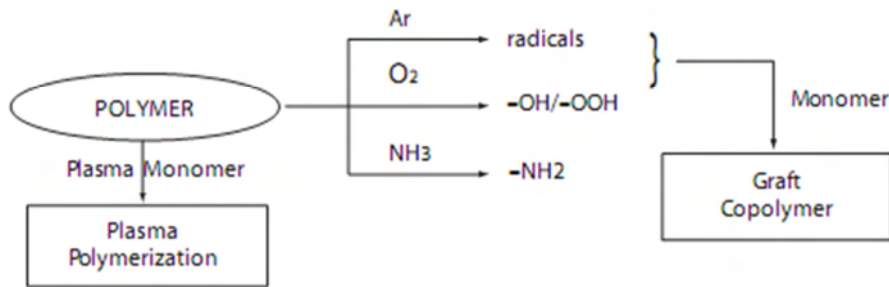
A concern with the high energy radiation techniques however is unwanted side reactions. Some of these include crosslinking, chain scission, post-irradiation degradation, discolouration, and long-term instability of the radiated products (Clough, 2001). Post-irradiation of PP has been well studied and high energy irradiation is known to lead to oxidative degradation of PP (Geuskens and Nedelkos, 1993; Mowery et al., 2007). Also gamma radiation requires a specialised facility that may not be easily accessible, and it uses a  $\text{Co}_{60}$  source which is a radioactive isotope, and hence is not environmentally friendly (Clough, 2001).

### 2.4.1.2 Plasma-induced graft polymerisation

Plasma modification uses a plasma source i.e. a gas in its ionised state, with an energy of 10-20 eV (Gupta and Anjum, 2003). The accelerated electrons from the plasma have sufficient energy to induce cleavage of the chemical bonds in the polymer surface and to form new functional groups, and free radicals, which subsequently initiate graft polymerization (Bhattacharya and Misra, 2004). Gases such as oxygen, helium, argon, carbon dioxide, and ammonia have been used as plasma sources. Depending on the gas used, different reactive groups can be

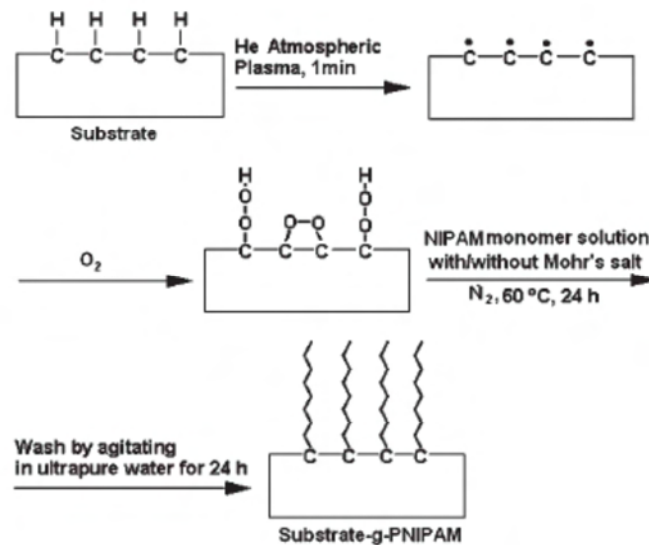
## CHAPTER 2: LITERATURE REVIEW

expected (**Scheme 2.3**). The reactive gases such as oxygen, ammonia, and carbon dioxide are used to create functional groups on the polymer surface such as peroxides, amino and carboxylic groups respectively while the inert gases create free radicals on the polymer surfaces, which are transformed into polar groups in the presence of air (Gupta and Anjum, 2003).



**Scheme 2.3:** Schematic showing process for plasma-induced graft polymerisation (Gupta and Anjum, 2003).

Atmospheric plasma has been previously used to graft PNIPAAm onto nylon and polystyrene surfaces by the peroxidation method whereby after plasma irradiation, the substrates were incubated in NIPAAm solution and heated to 60 °C (Wang and McCord, 2007). The proposed method of grafting is shown schematically in **Scheme 2.4**, and involves the formation of peroxide functional groups on the polymer surface which are very reactive to graft polymerisation.



**Scheme 2.4:** Schematic of graft polymerisation of PNIPAAm onto nylon or polystyrene surfaces using atmospheric plasma (Wang and McCord, 2007).

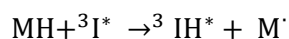
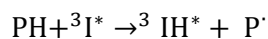
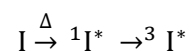
## CHAPTER 2: LITERATURE REVIEW

Plasma modification however is conventionally line-of-sight and modification is typically not extended to the bulk of the matrix. Vacuum plasma may penetrate a few microns into the surface and depending on the scaffold porosity it may be possible to extend the modification, however conventionally this technique is limited to surface modification only.

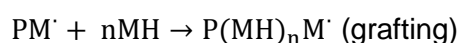
### 2.4.1.3 Photo-irradiation

Photo-irradiation or photo-grafting involves irradiating a polymer surface with a UV-light source (e.g. using a mercury lamp). The energies delivered are in the wavelength range of 200-400 nm and much lower than high-energy radiation, but the energies are still comparable to covalent bond energies hence enabling bond rupture and the formation of free radicals on the polymer backbone (Desai and Singh, 2004). Typically a photo-initiator also known as a chromophore is used (such as organic peroxides and organic ketones such as benzophenone). The mechanism for photo-grafting is given in **Scheme 2.5**.

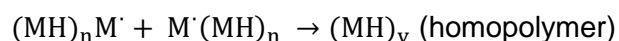
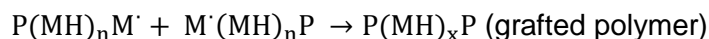
#### Initiation:



#### Propagation:



#### Termination:



**Scheme 2.5:** Mechanism of photo-grafting where I, PH, MH refer to the initiator, polymer, and monomer respectively (Desai and Singh, 2004).

The initiator (I) acts by absorbing the UV light during irradiation, whereby the molecule goes from the ground state to the first excited singlet state ( ${}^1I^*$ ), and then relaxes to the excited triplet state ( ${}^3I^*$ ) (Desai and Singh, 2004). The extra energy is dissipated in various pathways of which energy transfer to the polymer and monomer

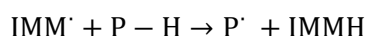
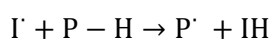
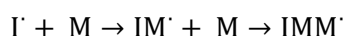
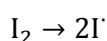
## CHAPTER 2: LITERATURE REVIEW

can occur; which can induce grafting and homopolymerisation. Grafting can proceed via hydrogen abstraction from the polymer backbone, creating a free radical on the polymer backbone ( $P'$ ) which acts as an active site for graft polymerisation (Desai and Singh, 2004). The initiator also abstracts a proton from the monomer creating a monomer radical ( $M'$ ) which can undergo graft polymerisation and homopolymerisation (Desai and Singh, 2004). Gueskens *et al.* grafted polyacrylamide and PNIPAAm onto PE using photochemical grafting with anthraquinone-2-sulfonate as the photo-initiator (Geuskens *et al.*, 2000). Photo-grafting however is limited to line of sight, and formation of large amounts of homopolymer can occur.

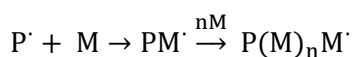
### 2.4.2 Graft polymerisation with chemical initiator

In this method a chemical initiator is used to form free radicals. Typically grafting proceeds as shown below in **Scheme 2.6** where I is the initiator,  $I'$  is the primary radical, P-H is the polyolefin backbone, and M is the monomer (Bhattacharya and Misra, 2004). Initiation proceeds as follows (1) dissociation of the initiator to form initiator radical species( $I'$ ), (2) addition of a single monomer molecule to the initiating radical ( $IM'$ ) and (3) formation of a free radical on the polymer backbone ( $P'$ ) Propagation and finally termination occur to produce the graft copolymer.

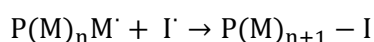
#### Initiation:



#### Propagation:



#### Termination:



**Scheme 2.6:** Typical mechanism for free radical induced graft polymerisation (Bhattacharya and Misra, 2004).

A number of chemical initiators can be used to initiate the grafting process and commonly include redox initiators or thermal initiators. Redox initiator systems



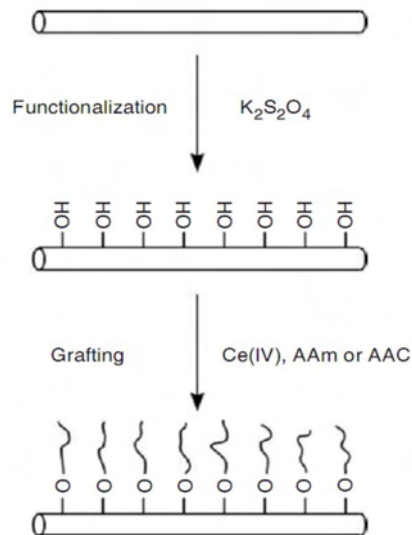
## CHAPTER 2: LITERATURE REVIEW

contain a reducing and oxidising pair whereby electrons are transferred from one reagent to the next, while thermal initiators have thermally labile bonds which can be activated by heating. Some of the chemical initiators which have been used for graft polymerisation for NIPAAm include peroxy sulphates; ceric ammonium nitrate; benzoyl peroxide; and other organic peroxides, which are activated in solution by heating to elevated temperatures (Gupta and Sahoo, 2001; Huang and Sundberg, 1995). Peroxy sulphates are one of the most widely studied initiator system for NIPAAm polymerisation. Peroxy sulphates such as ammonium persulphate (APS), sodium persulphate, and potassium persulphate can be activated by various means, either thermally, or it can be reduced by  $\text{Fe}^{2+}$  or diamines to sulphate free radicals. APS and tetramethylethylenediamine i.e. TEMED are a commonly reported redox system for polymerisation and graft polymerisation of NIPAAm at room temperature. TEMED is the reducing agent thereby acting as a catalyst or promoter and the rate of radical formation from APS is enhanced (Xinqiu et al., 1989).

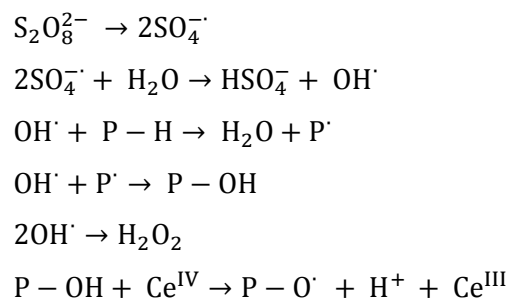
The primary radical produced from persulphates is  $\text{SO}_4^{\cdot-}$ . According to Riggs the thermal decomposition of APS yields both sulphate and hydroxyl radicals ( $\text{OH}^{\cdot}$ ) (Riggs and Rodriguez, 1967). There are different views regarding the reactivity of  $\text{SO}_4^{\cdot-}$ . Some authors report that  $\text{SO}_4^{\cdot-}$  can react directly with the polymer backbone to produce the polymer radical by H abstraction, while others report that the produced  $\text{OH}^{\cdot}$  is responsible for forming free radicals on the polymer (Bhattacharya and Misra, 2004). Still others report on a two stage graft polymerisation method using APS, whereby the polymer backbone (P-H) is firstly hydroxylated (P-OH) by thermal decomposition of APS (70-100 °C) and the hydroxylated backbone is then initiated with a transition metal such as ceric ion (Ce) to form the polymer radical (P-O $^{\cdot}$ ) (Bamford and Al-Lamlee, 1994).

A simple schematic of the functionalisation process is shown in **Scheme 2.7**. Due to its convenience, mild conditions, and aqueous medium, many studies have focused on the two stage method using APS/Ce (Amornsakchai and Doaddara, 2008; Curti et al., 2002; Curti et al., 2005; Zhao and Geuskens, 1999). The proposed mechanism of the 2 step process is given in **Scheme 2.8**.

## CHAPTER 2: LITERATURE REVIEW



**Scheme 2.7:** Schematic of graft polymerisation of acrylamide and acrylic acid onto a polyethylene backbone using persulphate and Ce (Amornsakchai and Doaddara, 2008).



**Scheme 2.8:** Proposed mechanism for polymer functionalisation using persulphate & Ce ion (Zhao and Geuskens, 1999).

Free-radical induced grafting by chemical initiation offers the advantage of being a simple method, which is easy to perform and which does not involve the use of high energies, and expensive equipment, however chemical initiators are required. Due to its simplicity and ease of use, free-radical induced grafting by chemical initiation was used in this study for development of the 3D PNIPAAm grafted NWF scaffolds. Furthermore fluorination was investigated for functionalisation of the NWF prior to graft polymerisation (**Chapter 5**).

### 2.4.3 Surface functionalisation by fluorination

It is known that the surface functionality of a polymer backbone influences free radical formation, as well as wettability and swelling of the polymer backbone in the graft medium. Swelling of the polymer is important to ensure mobility of the free radicals from the monomer to graft sites on the polymer backbone (Bhattacharya and Misra, 2004). Many studies have focused on improving functionality of polymer backbones using various surface functionalisation techniques. Methods which have been used include UV treatment, plasma treatment, gamma irradiation, ozone treatment, use of etching agents, chemical treatment, flame treatment, corona discharge, and fluorination (Curti et al., 2002; Curti et al., 2005; Zhao and Geuskens, 1999). This review focuses on fluorination as a surface functionalisation technique as it is cost-effective and less invasive than most of the other radiation techniques.

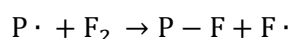
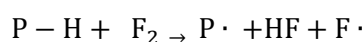
Fluorination, i.e. treatment of a polymer with elemental fluorine ( $F_2$ ) is an attractive surface functionalisation method. Fluorination involves bombarding the polymer surface with a  $F_2$  gas mixture (containing oxygen) in the dry state under mild conditions. Fluorination can be used to modify polymer articles of any shape (Kharitonov, 2000). Additionally  $F_2$  gas can penetrate polymer surfaces to large depth whereby the thickness of modification is within 0.01-10  $\mu\text{m}$  (Kharitonov, 2000). The depth of modification is a diffusion-controlled process, and the rate of formation of the fluorinated layer is controlled by the diffusion of molecular  $F_2$  gas through the fluorinated layer to the untreated polymer. This depends on the polymer nature,  $F_2$  partial pressure,  $F_2$  gas mixture, reaction time and temperature (Kharitonov et al., 2005). A further advantage of fluorination, is that due to the exothermic nature of the reaction, the reaction proceeds at room temperature in a low vacuum, with no heat, initiators, or catalysts required (Kharitonov et al., 2005).

Fluorination can be classified into two categories i.e. ordinary direct fluorination and oxyfluorination (Jeong et al., 2011). The former uses a mixture of  $F_2$  (1-20 vol. %) and an inert gas (such as nitrogen, argon or helium), whereas the latter employs a mixture of fluorine and  $O_2$ . It is known that commercial  $F_2$  gas contains trace amounts of  $O_2$ , and hence some authors have indicated that fluorination always accompanies oxyfluorination, while other authors have shown no oxygen containing groups in fluorinated samples (du Toit and Sanderson, 1999). Direct fluorination is typically used to improve the barrier properties of polymers by lowering the surface free energy, while oxyfluorination is commonly used to improve the adhesion properties of

## CHAPTER 2: LITERATURE REVIEW

polymers by increasing the polarity, surface energy, and wettability of polymers (Kharitonov and Kharitonova, 2009). Many studies have reported modification of polymer surfaces by fluorination and in recent years direct fluorination is being widely utilised for industrial applications such as barrier properties of automotive fuel tanks, and storage vessels for toxic solvents (Kharitonov, 2000; Kharitonov, 2008; Kharitonov et al., 2005).

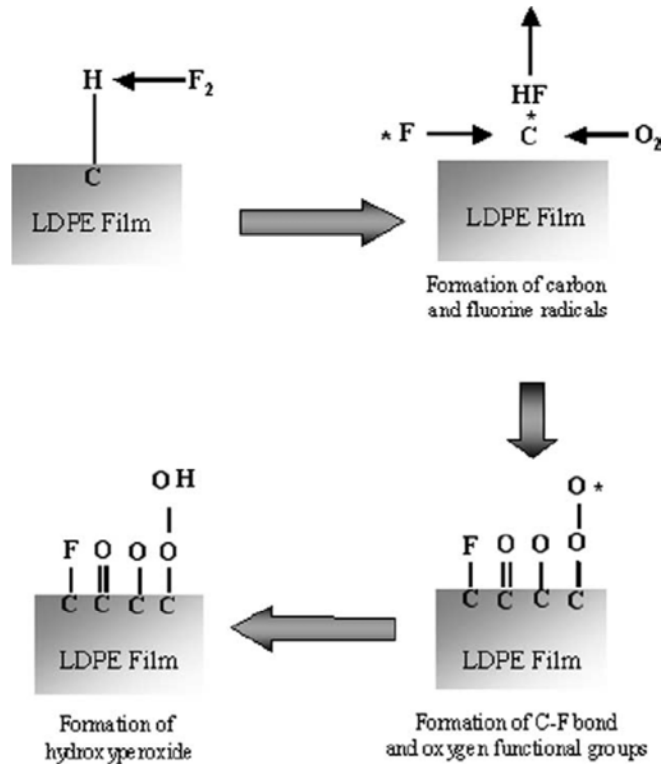
During fluorination,  $F_2$  reacts exothermically with the surface of a hydrocarbon by a free radical mechanism as shown in **Scheme 2.9** (du Toit and Sanderson, 1999). Due to its high electronegativity,  $F_2$  abstracts protons from a polymer (P-H) to form polymer free radicals ( $P^\cdot$ ), fluorine free radicals ( $F^\cdot$ ), as well as hydrogen fluoride. The process has also become very safe and reliable nowadays whereby excess  $F_2$  is neutralised and hydrogen fluoride is converted into the solid phase by e.g. using sodium fluoride pellets (Kharitonov and Kharitonova, 2009).



**Scheme 2.9:** Proposed mechanism for direct fluorination of polymers.

According to Kharitonov, direct fluorination of polymers results in disruption of -C-H and -C-OH groups and saturation of double bonds which is followed by formation of fluorinated groups such as -C-F, -CF<sub>2</sub>, and/or -CF<sub>3</sub> because of the higher bond energy of C-F bonds, compared to C-H or C-OH bonds (Kharitonov, 2008). During oxyfluorination, molecular O<sub>2</sub> reacts spontaneously with the fluorocarbon radicals generated by F<sub>2</sub> and oxygen and fluorine containing functional groups are formed (Kharitonov, 2000). A schematic representation of the process is given in **Figure 2.9**. Many authors have reported that acid fluoride (-COF) is hydrolysed to the highly polar carboxylic acid (-COOH) group (du Toit and Sanderson, 1999; Kharitonov et al., 2005; Lee et al., 2003). The formation of reactive peroxide (-O-O) groups and long-lived trapped peroxy radicals on oxyfluorinated polymer surfaces is also well-known (du Toit and Sanderson, 1999; Jeong et al., 2011; Kharitonov, 2000; Kharitonov et al., 2004). Tressaud *et al* demonstrated middle (-CH(OO<sup>•</sup>)- or -CF(OO<sup>•</sup>)-) and “end” peroxy groups (-CH<sub>2</sub>OO<sup>•</sup> or -CHFOO<sup>•</sup> or -CF<sub>2</sub>OO<sup>•</sup>) on oxyfluorinated low-density polyethylene (LDPE). It has been reported that the amount of peroxy radicals exceeds the amount of fluororadicals (Tressaud et al., 2007).

## CHAPTER 2: LITERATURE REVIEW



**Figure 2.9:** Schematic showing the oxyfluorination process (Park et al., 2005).

Despite the formation of peroxy groups on oxyfluorinated polymer, surprisingly the use of oxyfluorination as a pre-treatment prior to polymer grafting has been very limited. Recently Jeong *et al* reported for the first time graft polymerisation of methacrylic acid and styrene onto oxyfluorinated low-density polyethylene films by thermal activation of the peroxy groups and without the use of any external initiators (Jeong et al., 2011). Jeong coined the term “oxyfluorination-assisted graft polymerisation” (OAGP) technique to describe this facile two-step process. In this study PP, PET, and nylon NWF were grafted with PNIPAAm using the OAGP method. We investigate both oxyfluorination and direct fluorination as surface functionalisation methods prior to graft polymerisation.

### 2.5 Advances in cell culture

There is consensus in the literature that the environment in which cells are cultured *ex vivo* plays a critical role in the cells performance. Aspects such as proliferation, differentiation, metabolic activity, function and phenotype are directly influenced by the growth conditions which cells are subjected to. The ultimate goal for *in vitro* cell

## CHAPTER 2: LITERATURE REVIEW

culture is to maintain the cells in their native state such that the cell behaviour and function is similar to the living tissue from which they were derived.

While the monolayer cell culture process is well-established and is currently the gold standard, it leaves a lot to be desired in terms of bridging the *in vitro* to *in vivo* gap. More and more researchers and larger pharmaceutical companies are now looking for alternative approaches to improve the *in vivo* predictive power of their cell cultures. As the fields of tissue engineering, regenerative medicine, drug screening and cell and genetic engineering are advancing, there is now a growing demand for an *in vivo*-like cell culture model which can be used to more accurately predict the cellular responses of living organisms (Pampaloni et al., 2007).

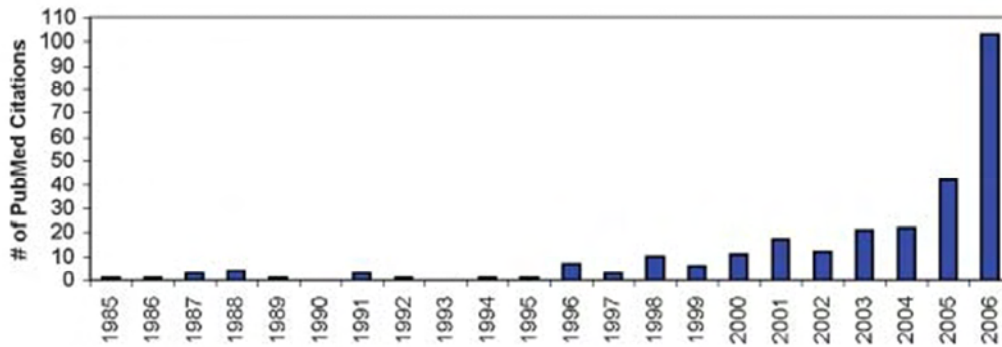
In recent decades advances have been made to the conventional cell culturing process, and these include the use of 3D scaffolds (see **Section 2.5.1**); PNIPAAm substrates (**Section 2.5.2**); and bioreactors (see **Section 2.5.3**).

### 2.5.1 3D scaffolds

Comparative studies between 2D and 3D cultures reveal that cells grown in 3D cultures display more relevant *in vivo*-like behaviour with respect to adhesion, cell morphology and extracellular matrix composition and function (Cukierman et al., 2001). Additionally vast differences have been observed in 3D cultures with respect to migration; differentiation; gene expression; metabolic activity; general cell function, molecular mechanisms, and drug metabolism compared to monolayer 2D cultures (Bokhari et al., 2007; Guillame-Gentil et al., 2010; Justice et al., 2009; Lee et al., 2008).

This decade has seen an exponential growth in the number of peer-reviewed articles published in the literature pertaining to 3D cell culture as shown in **Figure 2.10**. The field is currently undergoing a paradigm shift where researchers, academia as well as larger pharmaceutical companies, are now looking to 3D scaffolds to improve the predictive potential of their *in vitro* cell cultures. Due to the interest amongst researchers there is now a peer-reviewed website ([www.3Dcellculture.com](http://www.3Dcellculture.com)) solely dedicated to serve as a comprehensive list of published literature in the field of 3D cell culture.

## CHAPTER 2: LITERATURE REVIEW



**Figure 2.10:** Growth in the number of research publication in the field of 3D cell culture from Pub Med citations from 1985 to 2006 (Prestwich, 2007).

Areas of application where 3D cultures and in particular physiologically relevant cell characteristics are of importance includes tumour biology for the development of cancer drugs; stem cell differentiation; proteomics; genomics; engineering of human tissues and organs, and hepatotoxicology models for drug screening (Prestwich, 2008). It is known that hepatocytes rapidly de-differentiate in monolayer cultures while hepatospecific functions are maintained for longer periods in 3D cultures representing a more physiologically relevant model for drug evaluation (Prestwich, 2008).

Over the past several years a number of 3D scaffolds with various morphologies have been developed and these include microcarrier beads, hydrogels, sponges, nanofibers, and porous filter inserts. Some of the commercially available 3D scaffolds appear in **Table 2.2**. The vast majority of commercially available 3D scaffolds which are in common use for cell propagation and harvesting are based on biomimetic matrices such as ECM proteins or polysaccharides and include scaffolds such as Matrigel™; Extracel™; and AlgiMatrix™. The ECM-based scaffolds offer the advantage of possessing specific native proteins and growth factors which would enable integrin-ligand binding with cells thereby regulating the cell signalling pathways as well as providing 3D support for the cells (Tibbitt and Anseth, 2009). Furthermore, with the ECM based hydrogels and sponges, cells are easily encapsulated within the network to encourage 3D cell growth (Prestwich, 2007; Tibbitt and Anseth, 2009).

## CHAPTER 2: LITERATURE REVIEW

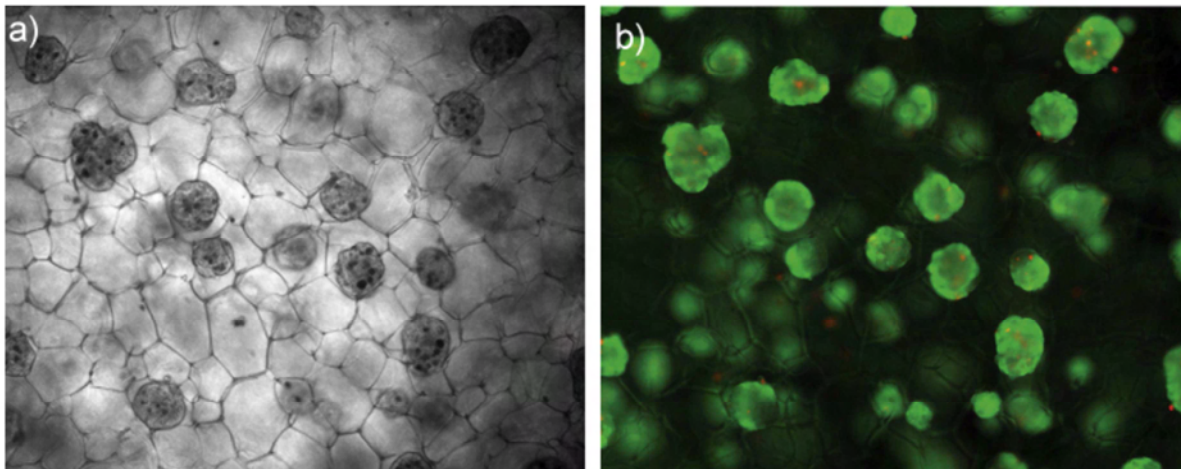
**Table 2.2:** Some of the commercial 3D scaffolds for cell growth and harvesting based on natural and synthetic materials (GE Healthcare, 2012; Justice et al., 2009; Prestwich, 2007).

| Scaffold trade-name   | Supplier                  | Form                             | Composition   |
|-----------------------|---------------------------|----------------------------------|---|
| Matrigel™             | BD Biosciences            | Hydrogel                         | Type IV collagen, laminin and heparin sulphate proteoglycan   |
| AlgiMatrix™           | Invitrogen                | Sponge                           | Alginate  |
| Extracel™             | Glosan Biosciences        | Covalently cross-linked hydrogel | Hyaluronan, and gelatine cross-linked with polyethylene glycol diacrylate   |
| Cytodex™              | GE Healthcare Biosciences | Microcarrier                     | Cross-linked dextran  |
| GEM™                  | Global Cell Solutions     | Microcarrier                     | Alginate core with a protein coat   |
| BD Biocoat™           | BD Biosciences            | Coated coverslips and trays      | Culture surfaces coated with ECM proteins and attachment factors  |
| BD PureCoat™          | BD Biosciences            | Coated coverslips and trays      | Culture surfaces coated with synthetic polymers such as Poly-L-Lysine   |
| Fibra-Cel®            | New Brunswick Scientific  | Non-woven fibre mesh             | Polyester mesh and polypropylene support  |
| Biomerix 3D scaffold™ | Synthecon                 | Foam                             | Polycarbonate-polyurethane urea   |
| Millicell®            | Millipore                 | Filter insert                    | Membranes based either on polytetrafluoroethylene; cellulose esters; polycarbonate and polyethylene terephthalate |
| Transwell®            | Corning                   | Filter insert                    | Collagen treated polytetrafluoroethylene  |



## CHAPTER 2: LITERATURE REVIEW

The current industry standard with more than 80% of the 3D cell culture market share is Matrigel™ (Prestwich, 2008). Matrigel™ is a reconstituted basement membrane collected from the Engelbreth–Holm–Swarm (EHS) tumour grown in mice and is uniquely suited for the culture of epithelial cells (Justice et al., 2009). Matrigel™ cell culturing involves reconstitution of purified basement membrane components in a specified ratio by gelation which is complete in 20 minutes (Tibbitt and Anseth, 2009). Matrigel™ has been shown to support natural cell growth, differentiation, angiogenesis, cell morphology and 3D cell behaviour (Kleinman et al., 1986; Prestwich, 2008). Extracel™ is a 3D cell culture product containing hyaluronic acid and gelatin which is prepared by seeding the gel with cells and then it is covalently cross-linked *in situ* under ambient conditions within 5-30 minutes (Prestwich, 2007). The crosslinking improves the stability of the matrix and improved batch to batch consistency has been shown (Prestwich, 2007; Prestwich, 2008). AlgiMatrix™ (**Figure 2.11**) is alternatively a ready-to-use alginate-based sponge, of an animal-free origin, and is fast becoming the scaffold of choice for primary and stem-cell spheroid culture but production variability is still an issue (Justice et al., 2009). AlgiMatrix™ is commonly used for 3D spheroidal culture as shown in **Figure 2.11**.

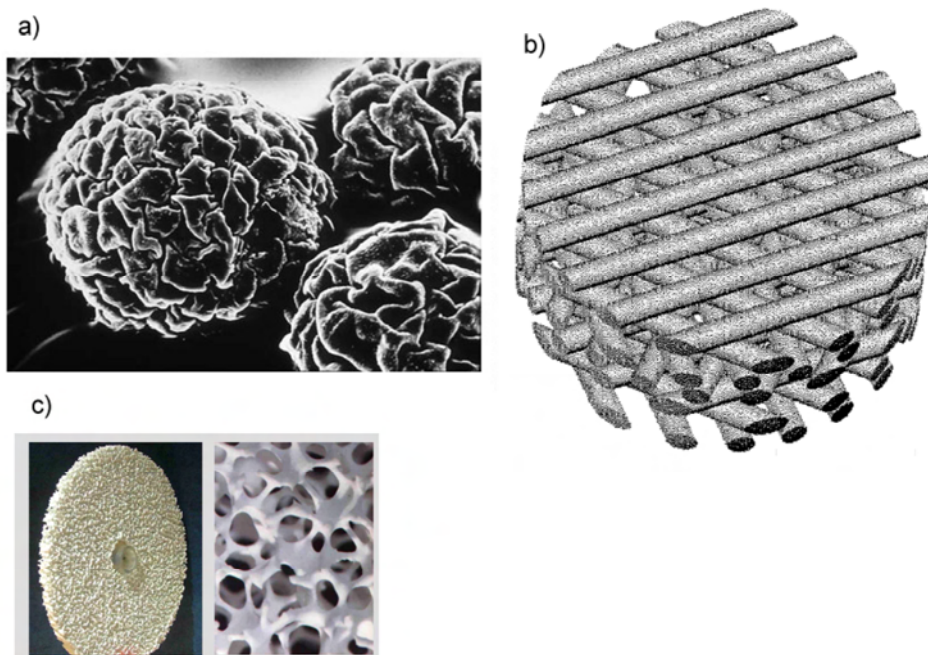


**Figure 2.11:** Images showing a) phase contrast microscopy; and (b) fluorescence microscopy showing human hepatocytes growing as spheroids when cultured in AlgiMatrix™ (live cells stained in green and dead cell appear red (Justice et al., 2009).

Microcarriers are promising alternatives to hydrogels for 3D cell culture in that it is more easily scalable by dispensing larger volumes per well and it is also cheaper (Justice et al., 2009). Microcarriers are small spheres, typically < 500 µm in diameter,

## CHAPTER 2: LITERATURE REVIEW

offering large surface area to volume ratios due to their unique curvature design thereby enabling high yield cell cultures. Microcarrier cultures can also undergo bead to bead migration, and scale-up is also possible without harvesting cells. In recent years, Cytodex™ and GEM™ microcarriers have been developed which are very attractive for high-density cell culture and can be used in 2D TCPS trays, or in bioreactors (**Figure 2.12**). Cytodex™ is a cross-linked dextran microsphere (~150 μm in diameter) which is transparent and displays a very large surface area (e.g. ~4400 cm<sup>2</sup>/g of dry weight for Cytodex 1) (GE Healthcare, 2009). Cytodex™ beads have also been developed with a thin layer of collagen (Cytodex 3) on the surface to enhance cell attachment. GEM™ i.e. Global Eukaryotic Microcarrier is a magnetic microcarrier with a gelatin coated alginate core for attachment to a wide variety of cells (Justice et al., 2009). The magnetic core allows for easy separation of the microcarriers during washing steps. An advantage of Cytodex is that various methods can be used to release adherent cell. This includes the use of dextranase which totally digests the microcarrier beads or collagenase to degrade the surface layer in Cytodex 3 (GE Healthcare, 2009).



**Figure 2.12:** Images showing (a) pig kidney cells growing on Cytodex 1 (GE Healthcare, 2009), (b) 3D Insert™ (3D Biotek) based on polystyrene fibre construct (Lui, 2008); and (c) highly porous Sponceram® disk (ZellWerk, 2012).

## CHAPTER 2: LITERATURE REVIEW

Other porous 3D scaffolds include synthetic materials such as 3D insert™ which are polystyrene and polycaprolactone fibre scaffolds, and Sponceram® disks which are highly porous hydroxyapatite disks with macro and micropores and a surface area of 14 m<sup>2</sup>/g (**Figure 2.12**).

Despite the growing popularity of the ECM based 3D scaffolds there still remain a number of challenges regarding its use in routine 3D culture. Some of these are listed below: (Justice et al., 2009; Prestwich, 2007):

- Poor reproducibility between batches of biomimetic scaffolds
- Lack of consistency between 3D cultures
- High pathogenic content and immunogenicity (Matrigel™ scaffolds)
- Some hydrogel scaffolds require preparation in-house to construct which is inconvenient and unreliable
- Limited ability to scale-up a single 3D format
- Post culturing processing and cell extraction difficult to handle
- High costs (Matrigel™ coated plates - \$100/plate)
- ECM based scaffolds have limited stability and specific storage requirements
- Need for higher throughput
- Better methods needed for characterising cells in 3D scaffolds
- Cell release methods (enzymes, or chemicals) can be destructive to cells

Based on these considerations, there is a growing need for new and improved 3D cell culture scaffolds.

### 2.5.2 PNIPAAm substrates for cell culture

Pioneering work in the early 1990's by Okano's group revealed for the first time a novel method to spontaneously attach and release cells in a manner which is harmless to the cells (Okano et al., 1995). Okano's research demonstrated that cells grown on PNIPAAm surfaces could be harvested non-invasively as an intact layer cell sheet containing deposited ECM (refer to **Figure 1.3** shown previously). Cell attachment and spontaneous cell release is possible on PNIPAAm surfaces, since it has been shown that cells can adhere and grow more easily on a PNIPAAm layer in its hydrophobic state, while cells release when the PNIPAAm layer becomes hydrated and hydrophilic (Yamada et al., 1990). With PNIPAAm cell culturing,

## CHAPTER 2: LITERATURE REVIEW

released cells remain intact with the only disruption being to the cell membrane i.e. between adhesive proteins on the basal side of the cultured cells and the polymer surface, while with the conventional enzymatic cell release methods, the cells extracellular membrane (ECM), cell-to-cell gap junctions, and membrane proteins such as ion channels and growth factor receptors are damaged (Canavan et al., 2005). Temperatures between 4-20 °C have been investigated for temperature induced cell release from PNIPAAm surfaces. The optimum temperature for cell release may differ for different cell types, but typically cell release at ~20 °C has been shown to be efficient (Matsuda et al., 2007; Okano et al., 1995; Yamato et al., 1999).

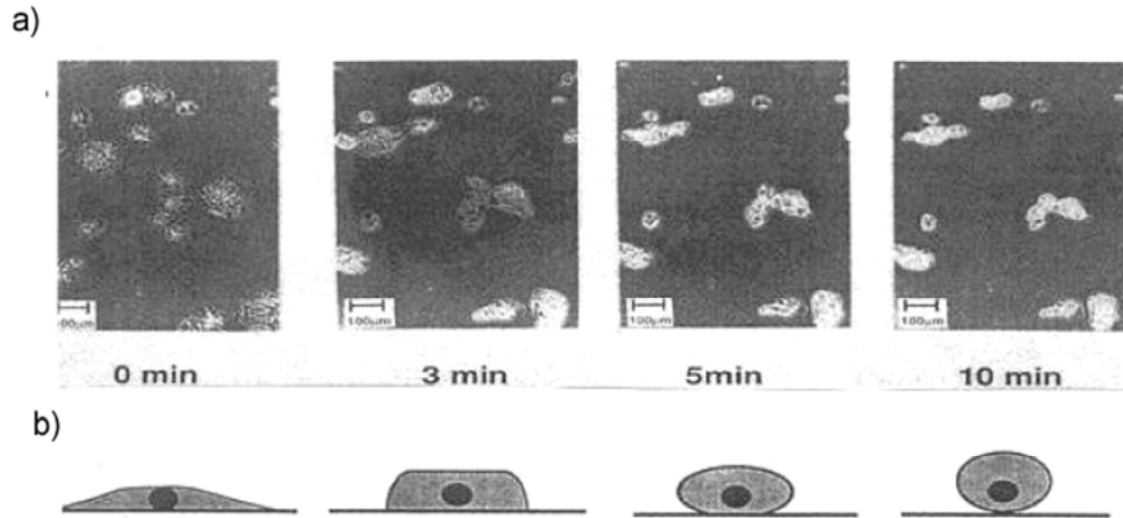
It is now well-accepted that PNIPAAm coated TCPS trays represents a viable scaffold for non-invasive cell culture. According to Hutmacher *et al* there was a 800% increase in the number of scientific articles published on PNIPAAm in 2004 compared to 1990 (data from Medline and Sciencedirect only) with the vast majority dealing with cell culture applications (Hutmacher, 2005). Recently as a spin-off of Okano's research, PNIPAAm coated trays, called Reppcell have recently been launched into the market by a company called CellSeed (Hutmacher, 2005).

The exact mechanism of temperature- induced cell release from PNIPAAm surfaces is still not fully understood since there are a number of factors which directly or indirectly influence the cell behaviour on these surfaces. These include the temperature of the cell culture medium; hydration/dehydration of PNIPAAm chains; protein adsorption/release from the surface; the PNIPAAm layer thickness and density of PNIPAAm chains; mechanical properties of the outer surface of PNIPAAm to influence cell spreading/contraction; and cellular metabolic activities requiring ATP consumption which influences the cell shape and cytoskeleton reorganisation (Cooperstein and Canavan, 2010 ; Kumashiro et al., 2010; Matsuda et al., 2007). The most well accepted mechanism for PNIPAAm-induced cell detachment is a two stage process as shown in **Figure 2.13** involving (1) passive detachment, and (2) active detachment (Cooperstein and Canavan, 2010 ).

“Passive detachment” involves liberation of cell adhesion molecules such as ECM proteins from the culture surface due to the hydration of the PNIPAAm. Cell attachment onto hydrophobic surfaces has been said to be mediated in part by the tight binding of ECM proteins (such as actin, and fibronectin) onto the surface. Rapid hydration of the PNIPAAm layer prevents anchorage of the ECM deposited on the culture surface, since physicochemical interactions (such as hydrophobic, columbic,

## CHAPTER 2: LITERATURE REVIEW

and van der Waals forces) between the cells and the surface are weakened (Yamato et al., 1999).



**Figure 2.13:** (a) Phase-contrast images of hepatocytes on PNIPAAm –TCPs trays during temperature-induced cell release at 0-10 minutes; and (b) a schematic representation of the mechanism of cell sheet detachment showing changes in cell contraction induced by an active cytoskeletal rearrangement (Scale-bar = 100 μm) (Cooperstein and Canavan, 2010 ).

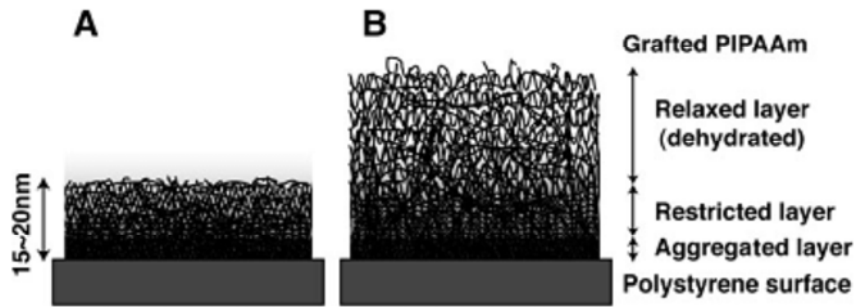
The second step is “active detachment” which is a cell metabolic process involving contractile forces which results in a change in cell shape from flattened and spread on cell adhesive surfaces to rounded and contracted on cell repellent surfaces (Kumashiro et al., 2010; Kushida et al., 1999; Yamato et al., 1999). The active cellular metabolic process involves intracellular signal transduction and reorganization of the cytoskeleton. During adhesion, pulling forces are generated by the cytoskeletal dynamics, which is in equilibrium with the centripetal traction forces mediated by stress fibres in the ECM on the culture surfaces. When the latter is lost due to hydration, the remaining tensile forces developed by the cytoskeleton cause cell rounding and complete detachment from the substrates (Yamato et al., 1999).

Immunoassay studies have shown that following low-temperature cell lift-off from PNIPAAm grafted surfaces, ECM proteins such as fibronectin, laminin, and collagen are detached from the tray surface together with the cell sheet, and functional cell-cell junctions are preserved in the cell sheets (Chen et al., 2005; Kushida et al., 1999). Cells grown on PNIPAAm surfaces also maintain highly differentiated functions compared to cells recovered by enzymes (Yamato et al., 2007).

## CHAPTER 2: LITERATURE REVIEW

It has been shown that the PNIPAAm graft density and thickness on TCPS also plays a critical role when developing reliable temperature-responsive surfaces. It is known that graft thickness affects the chain mobility of the PNIPAAm layer, which indirectly influences cell attachment and release. It has been reported that for reversible cell attachment and release, the chain mobility of PNIPAAm must be restricted to a certain extent. Studies conducted by Akiyama *et al* have demonstrated that endothelial cells only attached onto PNIPAAm trays of graft thickness  $15.5 \text{ nm} \pm 7.2 \text{ nm}$  ( $1.4 \pm 0.1 \mu\text{g}/\text{cm}^2$ ), whereas no cells attached on surfaces when the graft layer was higher ( $29.3 \text{ nm} \pm 8.4 \text{ nm}$ , and  $5 \mu\text{m}$ ) (Akiyama et al., 2004). This study reports an optimum PNIPAAm graft thickness of  $\sim 15\text{-}20 \text{ nm}$  (Akiyama et al., 2004), however this is in disagreement with other reported works (Kumashiro et al., 2010; Mizutani et al., 2008). Mizutani *et al* used a controlled polymerisation technique to produce PNIPAAm layers with well-defined PNIPAAm thicknesses ranging from  $1.8 \text{ nm}$  to  $64.7 \text{ nm}$  (Mizutani et al., 2008). This study revealed that cell adhesion increased as the PNIPAAm layer thickness decreased, and was still possible on very thin PNIPAAm layers. Cells detached as a complete monolayer at  $20 \text{ }^\circ\text{C}$  (for 1 hour) where the PNIPAAm thickness was  $10.9 \text{ nm}$  which is thinner than the conventional PNIPAAm coatings developed by Okano. This discrepancy has been attributed to differences in the coating microstructure amongst the studies, as well as a lack of understanding of the interfacial interactions with cells (Cole et al., 2009).

According to Matsuda *et al*, depending on the PNIPAAm thickness, different chain mobilities are possible at the outermost PNIPAAm surface which influences cell attachment and release as shown in **Figure 2.14** (Matsuda et al., 2007). It has been postulated that ultrathin PNIPAAm coatings display an aggregated surface layer and are unable to overcome the strong hydrophobic interactions at the PNIPAAm – TCPS interface resulting in insufficient hydration at  $T < \text{LCST}$  and cells are unable to efficiently detach (Akiyama et al., 2004; Matsuda et al., 2007). Conversely very thick coatings display a relaxed surface layer and enhanced chain mobility and hydration of the PNIPAAm chains, whereby cells cannot effectively attach at  $T > \text{LCST}$ . For reversible cell attachment and release, it has been reported that a restricted surface layer is required, such that the hydration and dehydration states are not excessive such that a quick interchange between both states is possible.

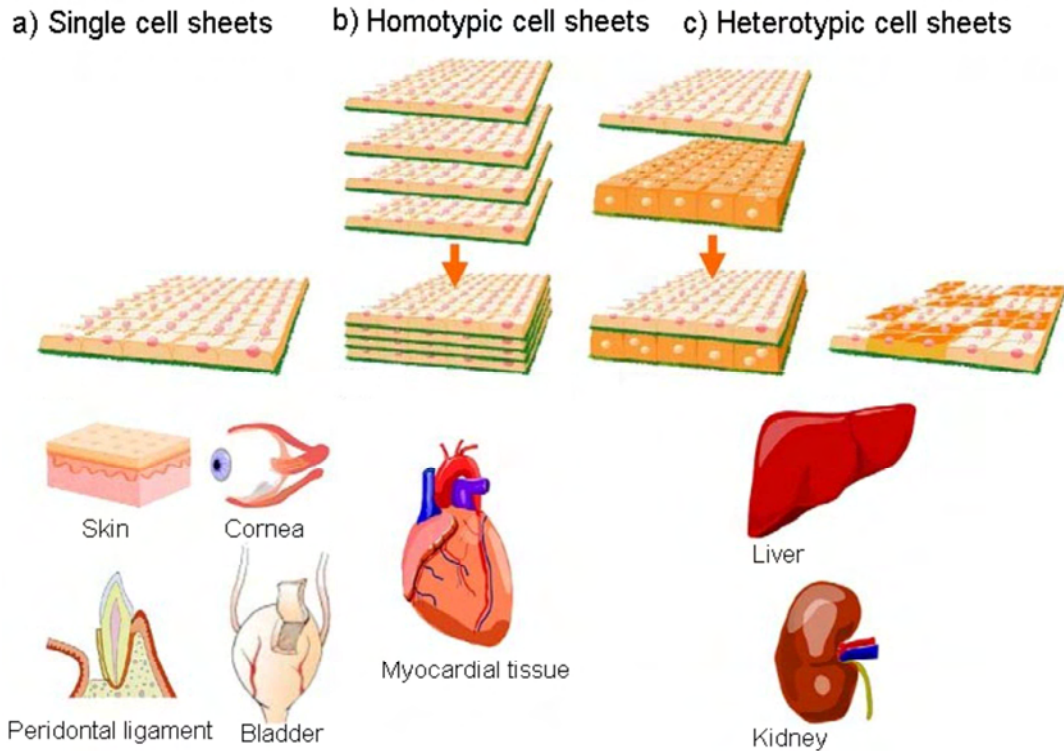


**Figure 2.14:** Image showing influence of graft thickness on chain mobility whereby graft thicknesses of 15-20 nm was found to be optimum for reversible cell attachment and release due to the presence of a restricted surface layer (Matsuda et al., 2007).

### 2.5.2.1 Cell sheet engineering

One of the major innovations in PNIPAAm cell culture has been in the area of cell sheet engineering for regenerative medicine. Cell sheet engineering refers to layering of individual cell sheets to create functional 2D or 3D tissues (Yang et al., 2005) (**Figure 2.15**). Numerous review articles have been published in the recent decade on this subject (Cole et al., 2009; Cooperstein and Canavan, 2010 ; da Silva et al., 2007; Kumashiro et al., 2010; Matsuda et al., 2007; Yamato et al., 2007; Yamato and Okano, 2004; Yang et al., 2005). Some of the advances in this field include multiple layering of heterotypic cell sheets e.g. hepatocyte cell sheets and endothelial cell sheets to create larger liver constructs (Harimoto et al., 2003). Patterned PNIPAAm surfaces with copolymers and non-cell-adhesive domains have also been developed to enable in situ co-cultures of heterotypic cells and recovery of co-cultured cell sheets for applications in regenerative medicine (Yamato et al., 2007). PNIPAAm cell sheet engineering has now been successfully applied to a variety of cell types including endothelial cells, corneal epithelial cells, chondrocytes, fibroblasts, keratinocytes, smooth muscle cells, hepatocytes, cardiomyocytes and various other stem cells (Moran et al., 2007; Murakami et al., 2006a; Nishida et al., 2004; Ohashi et al., 2007; Shimizu et al., 2002).

## CHAPTER 2: LITERATURE REVIEW



**Figure 2.15:** Image illustrating the concept of tissue reconstruction using cell sheet engineering whereby (a) single cell sheets (b) homotypic layering of cell sheets; (C) heterotypic cell sheet layering, and co-cultures from patterned surfaces can create lower and higher-order structures such as skin, cornea, myocardial tissue, kidney and liver (Yang et al., 2005).

Cell sheet engineering displays a number of advantages over direct cell injection or tissue engineering using biodegradable scaffolds (Yang et al., 2005). These include:

- The need for mild temperatures for spontaneous cell detachment
- No need for enzymes or harsh release methods
- Cells are released as an intact cell sheet together with the ECM proteins and cell-cell connections which are required for functional tissue
- Detached sheets can be used directly as an implant material in a host without requiring sutures, or without containing any foreign material which could induce an inflammatory response
- Possible to construct cell dense structures such as heart or liver
- Possible to develop large cell constructs without limitations of passive oxygen diffusion and loss in viability in the core of the construct



## CHAPTER 2: LITERATURE REVIEW

One of the challenges of this technology is cell sheet contraction upon release. Supporting membranes based on poly(ethylene terephthalate) and poly(vinylidenedifluoride) have been used to manipulate cell sheets without contraction (Matsuda et al., 2007). A recent paper reports the use of a PNIPAAm surface that was treated with oxygen plasma treatment to produce cell sheets on thin insoluble PNIPAAm layers with improved stability and storage (Shimizu et al., 2010).

While PNIPAAm cell sheets serve as a promising tool for engineering tissue, a limitation of the current technology is that it is primarily based on the use of flat 2D substrates which lacks structural and organisational cues for cells (Isenberg et al., 2008). Where layered or patterned co-culture cell sheets are used the process requires multiple steps and does not address the need for a structural matrix to enable cell growth in three dimensions.

### 2.5.2.1 3D PNIPAAm scaffolds for 3D cell culture

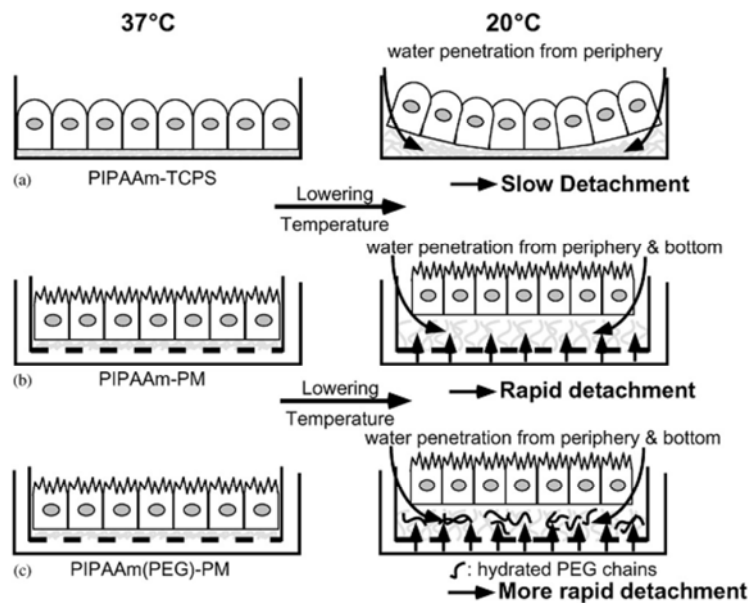
In recent years some studies have been reported with respect to development of porous PNIPAAm scaffolds, however for the majority of the scaffolds, the focus is still on the growth and release of 2D cell monolayers, whereby the pores are submicron and serve mainly for ease of hydration, oxygenation and nutrient supply to cells. Some of the recent studies are discussed below:

#### a) Porous PNIPAAm membranes and textured trays

Cell detachment from conventional PNIPAAm grafted TCPS trays is known to be slow since hydration is the rate limiting step as it occurs from the periphery of the PNIPAAm layer to the centre. Kwon *et al* developed a porous PNIPAAm membrane for cell culture whereby PNIPAAm was grafted onto PET membranes (with pore size of 0.45  $\mu\text{m}$ , pore density =  $1.6 \times 10^6 / \text{cm}^2$  and surface area =  $4.2 \text{ cm}^2$ ) using electron beam irradiation (Kwon et al., 2000). The PET-g-PNIPAAm porous membranes displayed enhanced cell detachment of primary bovine aortic endothelial cells, whereby only 30 min was required to detach monolayer 2D cell sheets from the porous membrane as compared to 75 minutes for cell sheet detachment from PNIPAAm-g-TCPS trays. Enhanced cell detachment was attributed to improved hydration of the PNIPAAm layer from both the periphery as well basal side of the membranes as shown in **Figure 2.16**. Further acceleration of cell detachment was

## CHAPTER 2: LITERATURE REVIEW

possible by grafting polyethylene glycol (PEG)-PNIPAAm copolymer onto the porous PET membranes (Kwon et al., 2003). PEG was used to increase the hydrophilicity of PNIPAAm to increase the wetting and diffusion of water molecules, and the swelling/de-swelling characteristics of PNIPAAm. The use of 0.5 wt % PEG in the PNIPAAm-co-PEG copolymer, which was then grafted onto the PET porous membranes, decreased the cell detachment time to 19 minutes i.e. almost half the time which was required for the previously reported PNIPAAm-g-PET porous membranes (without PEG).



**Figure 2.16:** Illustration of cell detachment from (a) PNIPAAm-g-TCPS trays, (b) porous PNIPAAm grafted membranes, and (c) porous PNIPAAm-co-PEG grafted membranes whereby hydration occurs from the periphery and the basal side of the porous membranes via the pores, and the use of PEG further enhances the hydration process (Kwon et al., 2003).

Murakami *et al* used the porous PNIPAAm grafted PET membranes to investigate the expression of a differentiated phenotype when culturing primary canine oral mucosal epithelial cells without the use of xenogeneic factors (Murakami et al., 2006b). This study showed that when epithelial cells were grown on porous PNIPAAm grafted inserts, keratin expression, and stratified epithelial layers were possible and similar to when the xenogeneic factors were used, which was not possible on the rigid PNIPAAm coated trays. This was attributed to the porous structure of the inserts which enabled apical and basal supply of nutrients to the cells.

## CHAPTER 2: LITERATURE REVIEW

Okamura *et al* grafted PNIPAAm onto polypropylene (PP) membranes containing antibodies using plasma-induced polymerisation for selective cell separation of a specific cell type (Okamura et al., 2008; Okamura et al., 2005; Toshiyuki and Midori, 2006). Monoclonal antibodies were adsorbed onto the PP-*g*-PNIPAAm membranes at 37 °C due to hydrophobic interactions, and enabled binding of targeted cells due to the antigen-antibody complex, while at 4 °C, cell release of the targeted cells was possible due to the hydration of the PNIPAAm chains (Okamura et al., 2008). Nonwoven PP membranes (average pore radius, 10 µm) were used in this study, and this study did not focus on cell expansion and propagation in 3D.

To provide cells with a defined structural organisation, Isenberg *et al* developed micro textured TCPS trays, which were grafted with PNIPAAm. The patterns on the TCPS trays, consisted of alternating grooves and ridges (50 µm wide, 5 µm deep). Vascular smooth muscle cells cultured on the PNIPAAm grafted micro textured trays, produced intact monolayer cell sheets consisting of cells that exhibited strong alignment in the direction of the micro pattern (Isenberg et al., 2008). An optimum range of PNIPAAm grafting density for the micro patterned TCPS with this method was found to be 45–50% that is slightly lower than what is typically used to graft TCPS culture dishes (53–55%) (Isenberg et al., 2008). Cells still grew in monolayers.

As mentioned previously most of the studies discussed above focus on the culture and release of 2D cell monolayers even when 3D PNIPAAm scaffolds have been used. Recently Duarte *et al* presented the first report on the development of a 3D thermoresponsive scaffold for 3D cell expansion and proliferation (Duarte et al., 2011). The scaffold used in this study is a poly(D,L-lactic acid) foam containing PNIPAAm with mean pores of 138 µm. However the release of 3D cellular structures and high-density cells was not reported.

### *b) Hydrogels*

It has been reported that bulk linear PNIPAAm and conventional cross-linked PNIPAAm hydrogels do not support cell attachment at 37 °C (Akiyama et al., 2004; Haraguchi et al., 2006). The lack of thermoresponsiveness of bulk PNIPAAm surfaces can be attributed to the increased hydrophilicity, and hydration of thick PNIPAAm layers and high chain mobility which prevents cell adhesion even at 37 °C (Akiyama et al., 2004). Many studies have been conducted to copolymerise NIPAAm with other monomers to form 3D PNIPAAm hydrogels for use in cell culture, but with

## CHAPTER 2: LITERATURE REVIEW

only limited success to date. Matsuda's group developed various thermoresponsive PNIPAAm hydrogels copolymerised or grafted with gelatin (Ohya et al., 2005; Ohya et al., 2001b), hyaluronic acid (HA) (Ohya et al., 2001a), and PEG (Kwon and Matsuda, 2006) to more closely replicate the ECM. For the PNIPAAm-*g*-gelatin hydrogels it was found that adhesiveness of human umbilical vein endothelial cells increased with an increase in the PNIPAAm: gelatine ratio, while the PNIPAAm-*g*-HA hydrogels were non-cell adhesive. It was found that cell adhesiveness was influenced by the stiffness of the hydrogel and its ability to withstand the cell traction forces during spreading.

Haraguchi *et al* describes the development of PNIPAAm-clay nanocomposites with improved mechanical properties. Various cell types were tested i.e. hepatoma cells, dermal fibroblasts and umbilical vein endothelial cells and in each case cells adhered and proliferated on the PNIPAAm-clay nanocomposite hydrogels regardless of the thickness of the gel, while little adhesion and no proliferation were observed on pure PNIPAAm hydrogels (Haraguchi et al., 2006). Complete cell sheet detachment was also achieved. The authors attributed improved cell attachment and proliferation on polymer/clay hydrogels to enhanced protein absorption due to surface ionic charges contributed by the exfoliated clay, and the balance of hydrophobicity (due to PNIPAAm chains) and hydrophilicity (due to hydrophilic clay) (Haraguchi et al., 2006).

### 2.5.3 Cell culturing in bioreactors

It is clear that in order to develop a reliable *in vitro* cell culture system, cells must exist in a dynamic flux environment similar to the dynamic state of living tissue. In recent years there has been increasing interest in the use of bioreactors for high density and more physiologically relevant cell cultures. A bioreactor is typically a closed contained vessel in which cells are cultured on a scaffold whereby the flow of the cell culture medium containing the relevant growth factors and nutrients is controlled and the cells are oxygenated. Bioreactors can also enable control and monitoring of various parameters such as pH, conductivity, CO<sub>2</sub>/O<sub>2</sub> levels, glucose consumption etc.

Bioreactors have been specifically developed to overcome the mass-transport diffusional limitations in static 2D cultures and to enable high-density cell cultures. It

## CHAPTER 2: LITERATURE REVIEW

is known that one of the main limitations with 3D cell cultures is the higher oxygen requirements for cell dense constructs. This particularly becomes a constraint for large scale mammalian culture such as in the case of tissue engineering and production of cell culture products. Oxygen has a poor diffusion capacity and solubility in static aqueous solution at physiological temperatures. It has been established that *in vitro* static systems, oxygen and nutrient supply to cells is limited to a distance of 100-200  $\mu\text{m}$  by passive diffusion from the fluid-scaffold interface (Volkmer et al., 2008). Studies have indicated that with cell constructs greater than  $\sim 200 \mu\text{m}$  grown in a static system, cells only survive at the periphery while the cells in the centre suffer from hypoxia (i.e. lack of oxygen supply), causing necrosis (i.e. cell death) at the centre of the constructs (Volkmer et al., 2008). This is often the limiting factor in scaling up 3D cell cultures. Perfusion bioreactors that perfuse culture medium directly through the pores of the scaffold have been found to be very efficient in reducing diffusional limitations by enhancing media transport at the construct periphery as well as within the internal pores, minimizing mass-transport limitations (Wendt et al., 2009). Bioreactors also enable enhanced oxygenation to cells by the use of external spargers or directly through the use of hollow fibres.

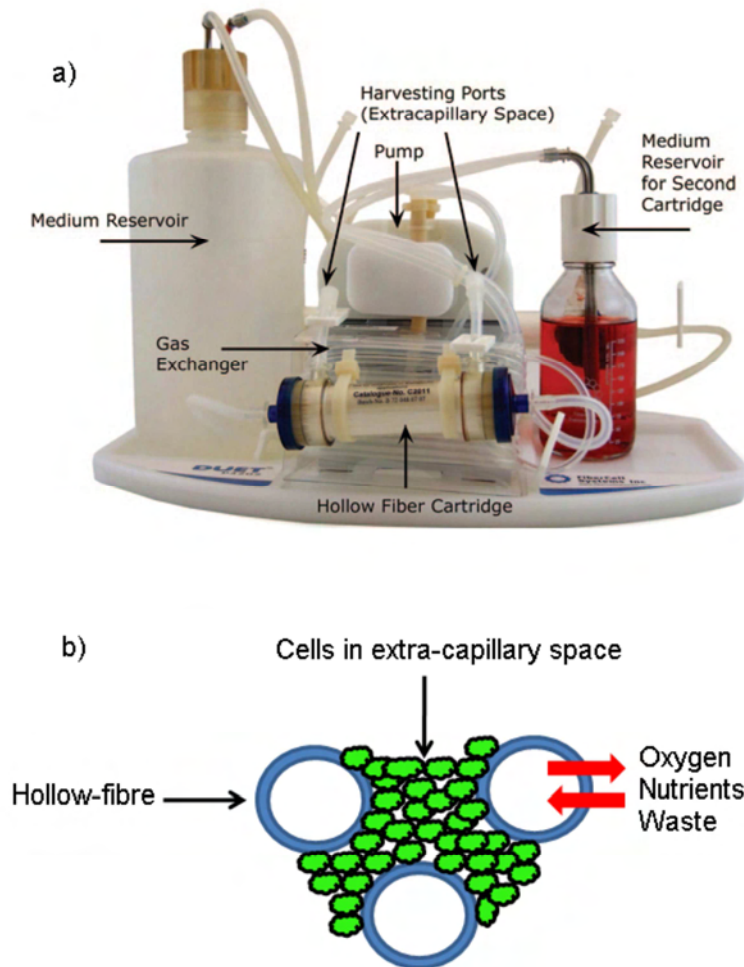
Bioreactors also very importantly enable fluid-driven stimulation of cells. Dynamic conditioning of cells is of particular importance in the case of cartilage, bone, cardiac tissue etc. where bioreactors have been used to apply mechanical stimulation such as stress-shielding and differential pressure to enhance the *in vitro* engineering of tissue (Wendt et al., 2009).

A number of bioreactors are in common use for cell culturing of anchorage-dependent cells, and these include stirred tank bioreactors, fluidised-bed, fixed-bed, ceramic matrix bioreactors, membrane bioreactors, spinner-flasks and hollow-fibre membrane bioreactors (Frost & Sullivan, 1999). The scaffolds used in these bioreactors are variable and often include microcarrier beads, microporous spheres, ceramic particles, membranes, and/or hollow fibres. Microcarrier cell culture in stirred tanked bioreactors is the most commonly used culture method for large scale culture of anchorage-dependent cells (Frost & Sullivan, 1999; Chu and Robinson, 2001; Hu and Aunins, 1997).

The most common use of bioreactors in cell culture is in the production of cell products such as proteins and monoclonal antibodies, and not necessarily for harvesting and releasing high density cells. In recent years the use of hollow-fibre

## CHAPTER 2: LITERATURE REVIEW

bioreactors are growing in demand for cell culture and are becoming increasingly popular for the production of monoclonal antibodies (Ye et al., 2006). Hollow-fibre bioreactors consist of networks or large bundles of hollow fibres with very small diameters (e.g. 200  $\mu\text{m}$  in the case of FibreCell Hollow fibre Systems), embedded in a cylindrical housing. The hollow-fibre bioreactor contains inlet and outlet ports at the ends of the cartridge to enable flow of liquid through the inner cavity of the fibres (inner lumen), while ports present on the top of the housing allows access to the area outside the fibres i.e. the extracapillary space (Cadwell, 2004; Ye et al., 2006) as shown in **Figure 2.17**.



**Figure 2.17:** (a) Image of FiberCell hollow fibre system (FiberCell Systems Inc, 2012) and (b) schematic drawing of a hollow-fibre membrane bioreactor used in cell culturing.

Generally the cells are grown in the extracapillary space where they can attach onto the fibres and grow in clusters, while the culture medium and/or oxygen is circulated through the inner lumen of the hollow fibres. The nutrients and oxygen diffuse through the membranes to the cells, while the metabolic waste diffuse away from the

## CHAPTER 2: LITERATURE REVIEW

cells into the inner lumen according to hydrostatic pressure difference and concentration gradients. This creates a nutrient circulation system similar to the capillary system in native tissue (Frost & Sullivan, 1999; Ye et al., 2006). A large number of commercially available bioreactors are based on hollow-fiber systems and include Primer HF; MiniMax; Maximizer; Excellerator; CellMax®; and FibreCell Systems.

Bioreactors offer several advantages when compared to static culture and include the following (Cadwell, 2004; Wendt et al., 2009):

- High-density cell cultures can be achieved and cells can grow in densities similar to that found *in vivo* i.e.  $> 10^8$  cells/ml as compared to standard cell culture (with spinner flasks) where only  $10^6$  cells/ml are possible
- Dynamic cell seeding is possible to reduce the inhomogeneity of static cell seeding
- High level of supply of oxygen and nutrient to cells reducing diffusional limitations
- Cells are physically stimulated by the shear forces which can induce signal pathways which is not possible in static growth conditions
- Control, monitoring and regulation of key environmental parameters such as temperature, pH, gas composition, humidity etc.) are possible
- Continuous or semi-continuous replenishment of spent media is possible with feedback mechanisms ensuring that the homeostatic of cells is maintained and not disrupted as is the case with manual media changes.
- Systems are automated and semi-automated to reduce the human intervention required which lowers contamination and improves the reliability and reproducibility of cell culture

Automated cell culturing systems typically involve culturing cells under controlled environmental conditions, while providing the capability of monitoring cell growth and maintaining media conditions (i.e. media pH, dissolved oxygen content, nutrient and waste concentration, cell concentration and cell viability). A number of automated systems have appeared on the market with different levels of automation, and capabilities (**Table 2.3**). Systems such as Cellmate™, and AcCellerator™ involve the use of robotics to automate the traditional processes performed within manual cell

## CHAPTER 2: LITERATURE REVIEW

culture, i.e. cell seeding on culture trays/flasks, media changes, bottle gassing, cell sheet rinsing, trypsination and/or cell scraping (Kempner and Felder, 2002).

**Table 2.3:** Examples of commercially available automated cell culture systems.

| Supplier                       | Products             | Application  |
|--------------------------------|----------------------|--|
| Cytogration                    | Cytogration System   | Automated system with 2D Multi-well plates                     |
| Automation partnership (TAP)   | Cellmate™<br>SelectT | 2D roller-bottles and T-flasks<br>T-flasks & microtiter plates |
| RTS Life Science International | AcCellerator™        | Automated system stack-based culture of 2D T-flasks            |
| CRS Biodiversity               | -                    | Membrane well plates or microtitre plates                      |
| Hamilton                       | CellHost             | BioLeviator™ bioreactor with GEM™ microcarriers (3D)           |

Consequently the same results are achieved but with improved consistency, substantially lower contamination rates, minimum human intervention, lower labour and process costs, higher yield, higher efficiency and improved turn-around times. Scale-up is also easier on these automated robotic cell culture type systems in that no regulatory approval required since the process is the same as with manual cell culture (Kempner and Felder, 2002). Automated systems are now common place especially in pharmaceutical companies where high-volume cell culturing is required on a routine basis. In recent years, the innovative BioLeviator™ has been developed which is a small bench-top device for high density disposable microcarrier cell culture as shown in **Figure 2.18**.

Although the current bioreactors and automated cell culture systems offer major advantages to the traditional manual cell culture system, not all key issues facing anchorage-dependent cell culturing are currently being addressed (i.e. use of 3D scaffold and non-invasive cell release). Additionally very few systems offer ease of operation (i.e. seeding of cells in the bioreactor, perfusion of the cell culture and maintenance of the cell culture through monitoring and medium exchange, and harvesting of the cell culture to be analysed) and monitoring and control of the



experimental parameters. Also the traditional automated systems have been large bulky devices.



**Figure 2.18:** The BioLevigator™ - a compact bench-top device with controls (Justice et al., 2009).

## 2.6 Conclusions

PNIPAAm is the most well-known and studied thermoresponsive smart polymer. PNIPAAm is mainly being explored for use in cell culture due to its mild phase transition conditions. PNIPAAm enables spontaneous cell release from its surface by merely cooling the media and without requiring any destructive methods. PNIPAAm hydrogels display challenges with respect to slow response time and poor mechanical properties. Studies have also shown that bulk PNIPAAm hydrogels is not effective as cell culture scaffolds due to the dense skin layer. However PNIPAAm grafted TCPS trays has proven to be an attractive technology for monolayer cell culturing. Various techniques are available for graft polymerisation of PNIPAAm onto polymer surfaces with chemical initiation being the simplest and most cost-effective option. A polymer surface must also preferably be functionalised with reactive groups to enhance grafting. Fluorination is an attractive functionalisation method since it is a dry technology, it is less invasive than other radiation methods, and it can form reactive polar groups on a polymer surface at room temperature.

With respect to cell culture, it is well-known that 3D culture is far superior when compared to 2D monolayer culture. Many 3D scaffolds are available for 3D cell

## CHAPTER 2: LITERATURE REVIEW

culture, however many of the scaffolds still rely on destructive means to release the confluent cells. To date only limited studies have focussed on the development of a 3D porous PNIPAAm scaffold whereby such a scaffold could be used routinely as a 3D cell culture insert for the release of 3D cell clusters and in a bioreactor for high-density 3D cell cultures. Although some attempts have been made at developing 3D PNIPAAm scaffolds such as porous membranes, hydrogels and micro textured surfaces, studies have focused primarily on the release of monolayers. Also bench-top bioreactors are being developed specifically for high-density 3D cell culture; however there still remains a gap with respect to enabling 3D cell culture together with non-destructive and non-invasive cell release of 3D cellular constructs. Wendt *et al*/ proposed in his review article on bioreactors that PNIPAAm could be used in a bioreactor for temperature-induced cell release, thereby eliminating the need for trypsin and the associated time-consuming processing steps, however to date no such system has been developed (Wendt *et al.*, 2009).

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