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Tunable Stimuli-Responsive Polymers for Cell Sheet Engineering

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1. Introduction

Anchorage dependent cells require a suitable substrate or scaffold for attachment, adhesion and growth. Conventionally cells are detached from substrates by mechanical or enzyme treatment, which can affect cell re-adhesion, viability etc. Poly (N-isopropylacrylamide) [PNIPAAm] is a well known temperature sensitive polymer with LCST of 32 °C and serve as a good substrate facilitating cell detachment without the use of proteolytic enzymes. Polymer and copolymers with N-isopropylacrylamide [NIPAAm] been proved to be excellent for cell detachment by temperature treatment. The co-monomers provide functional groups that bind to biological molecules and form hybrid polymer for enhanced cell function. This chapter reviews and compares different smart intelligent surfaces, method of preparation, characterization techniques and applications of cell sheet engineering. PNIPAAm based surfaces are able support cell growth and then to detach without any chemical reagent step. These thermo responsive substrates avoid the problem associated with scaffold based tissue engineering.

2. Cell sheet engineering

Tissue engineering involves ex vivo expansion of anchorage dependent cells in three dimensions to provide functional tissues (Langer and Vacanti, 1993). Prof. Robert Langer, Dr. Joseph and Dr. Charles Vacanti proposed the concept of tissue engineering in order to overcome the limitations of organ transplantation. Over the past 20 years, using the technology of tissue engineering, many kinds of tissues such as bone, heart valves, cartilage, corneal epithelium, cardiac muscles and skin have been successfully reconstructed (Yang *et al*, 2005; Nishida *et al*, 2004). Most anchorage dependent mammalian cells must adhere to a solid substrate in order to proliferate and manifest their function. The substrates used for cell growth should possess many features like the ability to encourage cell adhesion, spreading and proliferation. Also it is essential that the cells are easily detached from the substrate after it has grown. Conventionally enzymatic proteolysis of extracellular matrix (ECM) with trypsin or other proteolytic enzymes used to detach cells from substrates which can affect cell re-adhesion, cell viability etc (Kumashiro *et al*, 2010). Thermoresponsive polymers are of great interest in tissue engineering applications specifically in cell sheet detachment. The effect of these stimuli responsive polymers was first studied by Heskins

and Guillet in early 1960's (Heskins and Guillet, 1968). The smart behavior of these polymers is because of its change in molecular conformation in response to a temperature change in the surrounding environment. N-isopropylacrylamide [NIPAAm] is a well characterized thermo responsive polymer that shifts its phase properties above and below its lower critical solution temperature (LCST) of 32 °C. Above the LCST, polymer will be hydrophobic resulting in the attachment and growth of cells while below the LCST it turns hydrophilic enabling the detachment of cells as a single cell or as a uniform cell sheet. Since the cell sheet could keep the cell junctions and deposited extra cellular matrix along with it, physiological functions and bioactivity of the cell sheet are similar to the cells in tissue, which could provide potential application as an engineered tissue. Polymer and copolymers with NIPAAm has been proved to be excellent for cell detachment by temperature variation. The group of Okano and coworkers done a lot of studies in thermoresponsive, Poly(NIPAAm) based polymers and proposed a new approach in tissue engineering called cell sheet engineering (Yamada *et al*, 1990).

3. Mechanism of cell attachment and detachment from the thermoresponsive surfaces

Cells initially attach to a surface by a process called passive adhesion in which the adherence is due to the physicochemical interaction such as hydrophobic interactions, coulomb forces and van der Waal's forces. Then the cells bind to a specific site on adhesive protein by a process called active adhesion via a receptor protein called integrin. Conventionally enzymes are used to detach cells from substrates, resulting in the destruction of cell membrane proteins and ECM which can affect cell re-adhesion and viability. But cell detachment from thermoresponsive surfaces retain their cellular structure and functions in which cell detachment occurs in a two step mechanism via a passive step, followed by an active step. In the passive step cell detachment is induced by the hydration of PNIPAAm chains on the substrate whereas in active step change of cell shape and detachment from the surface is driven by cytoskeletal action and metabolic process (Okano *et al*, 1995).

4. Methods of preparation of temperature responsive intelligent surfaces

Thermoresponsive polymers can be grafted on different substrates such as silicon, glass, quartz, polyethyleneterephthalate sheets etc but commonly grafted on Tissue Culture grade Poly Styrene (TCPS). The choice of substrate, method of application of thermoresponsive polymer on the substrate and thickness of the grafted polymer has a major role on cell attachment and detachment phenomena (Elloumi-Hannachi *et al*, 2010). It is reported that a grafting thickness of 15 to 20 nm is a prerequisite for efficient cell attachment and detachment (Akiyama *et al*, 2004).

Many types of polymerization techniques can be used for grafting the thermoresponsive surfaces such as Electron beam irradiation, Gamma radiation, Plasma polymerization, UV irradiation and Atom transfer radical polymerization.

4.1 Electron beam induced polymerization

Electron beam polymerization (EB) is a popular method used for producing thermoresponsive surfaces for cell sheet engineering (Yamato *et al*, 2010). NIPAAm monomer is covalently bonded onto the TCPS (tissue culture polystyrene) dishes by the irradiation of electron beam

on to the monomer. Electron beam method facilitates even, thin grafting and the large scale production of temperature responsive culture dishes even though it is expensive. The thickness is controlled by monomer concentration and radiation energy (Nagase *et al*, 2009).

4.2 Gamma irradiation

Gamma radiation is another method for the grafting of thermoresponsive surfaces on TCPS (Akiyama *et al*, 2004; Anil Kumar *et al*, 2007). This study also revealed that grafting by gamma irradiation is comparable with conventional electron beam irradiation and facilitates batch processing of polymerization and grafting of PNIPAAm. Moreover it avoids most sophisticated equipment like electron beam accelerator. The efficacy of the grafted surface was confirmed by the successful growth of different cell lines such as L-929 (mouse subcutaneous connective tissue fibroblast), NRK-49F (normal rat kidney), SIRC (rabbit corneal epithelium), and HOS (human osteosarcoma) and detachment of the cell sheets while maintaining the cell-cell and cell-extra cellular matrix contact.

4.3 Plasma polymerisation

Plasma polymerization is a different single step method which facilitates the preparation of thermoresponsive coatings on a solid substrate. A plasma glow discharge of NIPAAm monomer vapour was used to deposit PNIPAAm onto solid surfaces such as silicon, glass or TCPS. The cell adhesion/detachment studies showed to be insensitive to the grafted layer thickness. But this method is not suitable for large scale production due to the difficulties related with continuous treatment and size (Canavan *et al*, 2005).

4.4 UV irradiation

UV irradiation is a yet another approach for the grafting of thermoresponsive coatings on to the solid substrate. Photopolymerisation and photografting of PNIPAAm is done on TCPS by UV irradiation (365 nm for 5 to 30 min) using photo initiators such as benzophenone (Nagase *et al*, 2009).

4.5 Atom transfer radical polymerization technique (ATRP)

ATRP facilitates the preparation of surfaces with dense polymer brushes from surface immobilized ATRP initiators (Mizutani *et al*, 2008). Preparation of PNIPAAm brushes on poly(4 vinyl benzyl chloride) coated TCPS surface using ATRP and application of these grafted surfaces to thermoresponsive cell culture substrates were studied by Mizutani *et al*. Attachment/ detachment process is favorable only in thinner surface less than 30 nm.

4.6 Reversible addition fragmentation chain transfer polymerization technique (RAFT)

RAFT polymerization technique allows the preparation of thermoresponsive PNIPAAm brushes with controlled chain length and graft density for cell sheet harvest. Chain length and density of PNIPAAm grafted on the surfaces have a significant effect on the thermoresponsive cellular behavior.

4.7 Oxygen plasma treated PIPAAm surface

Oxygen plasma treated PNIPAAm film is a promising material as a cell culture substrate in cell sheet engineering (Shimizu *et al*, 2010). A cast and dried film of thermoresponsive

PNIPAAm was fabricated and treated with high intensity oxygen plasma. This novel method of cell sheet has mechanical strength with high stability and low cost.

4.8 Solution casting method

Most of the above techniques are expensive and might not be reachable to all researchers. An auxiliary simple and cost effective approach is the coating of thermoresponsive polymer onto the TCPS by solution casting method. Thickness of coating obtained by this method is reported to be in micron level and astonishingly doesn't affect the cell attachment and proved to be efficient for cell sheet attachment and detachment process (Joseph *et al*, 2010; Varghese *et al*, 2010).

5. Different methods of characterization of thermoresponsive substrates

Different characterization techniques are employed for the physicochemical and biological evaluation of thermoresponsive substrates which is very important. Physicochemical characterization techniques such as Attenuated total reflectance Fourier Transform spectroscopy (ATR-FTIR), Nuclear magnetic Resonance spectroscopy (NMR) are used for the qualitative and quantitative detection, while Atomic Force Microscopy (AFM), Ellipsometry, surface Plasmon resonance, Profilometry are used for the determination of thickness of the grafting or coating.

The presence and quantity of PNIPAAm on the thermoresponsive surface can be determined by the technique ATR-FTIR (Akiyama *et al*, 2007). The presence of characteristic peak of amide carbonyl group around 1650 cm^{-1} showed the presence of NIPAAm. The amount of PNIPAAm on the surface of TCPS significantly affects cell adhesion behaviour. The quantity of grafted PNIPAAm could be detected by the ratio of peak intensities (I_{1650}/I_{1600}) in ATR-FTIR.

NMR technology also provides quantitative as well as qualitative assessment of thermoresponsive surface (Joseph *et al*, 2010). This techniques provides detailed structural information about the polymers and gives a better understanding about the polymerization mechanism.

Water contact angle measures the hydrophobicity and hydrophilicity of the surface and a contact angle of 70° proved to be optimal for cell sheet adhesion and detachment (da Silva *et al*, 2007). The thermoresponsive surface wettability changes of free end linear and multipoint attached PNIPAAm surfaces were investigated using the Wilhelmy plate technique. A large contact angle change was observed for an end grafted PNIPAAm surfaces whereas a small contact angle change for multipoint attached surfaces due to the restricted chain conformation of the multipoint attached grafted surfaces.

Differential scanning calorimetry (DSC) is widely used for the detection of LCST. The LCST of homopolymer PNIPAAm lies around 32°C and copolymers of PNIPAAm centered below or above 32°C depending upon the hydrophobicity and hydrophilicity of the comonomer.

Xray Photoelectron Spectroscopy (XPS) is one of the efficient methods for the qualitative assessment of the presence of PNIPAAm on different substrates (Jun and Okano, 2010) The elemental composition of carbon, nitrogen and oxygen on the grafted thermoresponsive surface, could be determined and should be in good agreement with the values based on the stoichiometry of the monomer, NIPAAm.

AFM can be used for the quantitative and qualitative characterization of thermoresponsive surfaces such as to observe the nano texture of the surface and to measure surface roughness and the thickness of the PNIPAAm coating (Jun and Okano, 2010). Profilometry is another method to visualize the surface morphology and the quantitative analysis of surface roughness and thickness from the surface profile measurement (Joseph *et al*, 2010). Determination of thickness from ellipsometry and surface plasmon resonance is very difficult because of the similarity in refractive index of both PNIPAAm and polymeric substrates (Nagase *et al*, 2009).

Biological evaluations of thermoresponsive surface is the most important characterization technique for cell sheet engineering and are done by cytotoxicity analysis, cell activity measurement, thermoresponsive efficacy, viability analysis and cell sheet characterization etc. The cytocompatibility, specific cytocompatibility, thermoresponsive efficacy studies are usually done using L929 cells, SIRC cells, HOS, NRK 49 F, Human fetal lung fibroblasts (TIG-1), primary cells from rabbit cornea, rat primary hepatocytes etc (Anil Kumar *et al*, 2007; Joseph *et al*, 2010; Varghese *et al*, 2010). Cells were maintained in MEM (Minimum essential medium) supplemented with 10% FBS (fetal bovine serum), 100 IU/mL penicillin and 100 µg/ml streptomycin at 37° C in a 95 % humidified atmosphere with 5 % CO₂. The cytotoxicity analysis can be done by using direct contact method, indirect contact method; High density polyethylene and zinc diethyl dithiocarbamate stabilized polyvinylchloride discs are usually used as negative and positive controls respectively. The cytotoxic evaluation is done by the comparison of the morphology of cells with positive and negative controls. MTT {(3-(4,5-dimethyl thiazol -2-yl) -2,5-diphenyl tetrazolium bromide} cell culture assay is usually used to evaluate the cell proliferation. Absorbance is measured using a multiwell plate reader at 540 nm. Cell viability is usually determined using neutral red staining. The thermoresponsive efficacy of the substrate is evaluated by retrieving the cell sheets by lowering the temperature below the LCST of the polymer and the viability of the retrieved cell sheet is determined using FDA (fluorescein diacetate) staining and observed under a fluorescence microscope. The cell sheet characterization is done by immuno staining technique using antibodies against proteins characteristic to tissue of interest.

6. Various thermoresponsive substrates for cell sheet engineering

Thermoresponsive substrates designed for cell sheet engineering have mainly used PIPAAm and its copolymers for cell adhesion and detachment. The use of thermoresponsive substrate to detach the confluent cell sheets without the use of conventional enzymatic treatments was first reported by Takezawa *et al* in 1990 (Takezawa *et al*, 1990). In this study they have used PIPAAm as a substratum by conjugating it with collagen for the culture of human dermal fibroblasts. These fibroblasts monolayer were harvested at reduced temperature by the dissolution of the dish coating. By the same time Yamado *et al* reported the successful culture of bovine hepatocytes on PNIPAAm grafted TCPS (Yamada *et al*, 1990). Homopolymer of NIPAAm has been reported to be grafted by various methods such as Electron beam polymerization, UV irradiation, Gamma irradiation etc and proved to be an excellent substrate for cell sheet engineering.

Copolymerization of NIPAAm with hydrophobic or hydrophilic monomer can modulate LCST for the systematic regulation of cell attachment and detachment. Thermoresponsive

copolymers based on N-isopropylacrylamide-Co-glycidylmethacrylate(NGMA), N-isopropylacrylamide-methylmethacrylate (NMMA), (Varghese *et al*, 2010) N-isopropylacrylamide-butylmethacrylate (Tsuda *et al*, 2004) and NIPAAm-MMA-phosphorylated HEMA (Thomas *et al*, 2010) were successfully used as a substrate for cell sheet harvesting. Thermoresponsive copolymer NGMA was clearly identified as potential substrate for cell culture harvesting system for generating 3D synthetic tissue. NGMA was synthesized by solution polymerization followed by the coating on TCPS surfaces by solution casting method. Copolymerisation of PIPAAm with the hydrophobic monomer glycidylmethacrylate results in a decrease of LCST. Thermoresponsive copolymer NMMA was also reported to be synthesized and coated by the same methodology mentioned above and proved to be an ideal thermoresponsive substrate with good cytocompatibility for cell culture and tissue reconstruction. Hydrophobic monomer n butylmethacrylate (BMA) has been copolymerized with PNIPAAm to reduce the LCST of NBMA in order to modulate the cell adhesion/detachment from culture dishes and revealed to be an efficient substrate for cell sheet engineering. Poly (N-vinylcaprolactum-co- N- isopropylacrylamide) PNVCL-co-PNIPAAm grafted on TCPS dishes by EB irradiation were efficiently applied to detach viable cell sheets from culture surfaces (Lim *et al*, 2007).

The spontaneous recovery of cell sheet is important for maintaining the viability of retrieved cell sheets. PNIPAAm grafted onto porous membranes facilitate the acceleration of cell sheet detachment by providing rapid water movement between the interface of cell sheets and membrane surfaces (Kwon *et al*, 2000a). Another method for the acceleration of cell sheet detachment is by cografing of a hydrophilic polymer Polyethylene glycol (PEG) with the thermoresponsive polymer PNIPAAm (Kwon *et al*, 2000b). Copolymerisation with 2-carboxyisopropylacrylamide (CIPAAm), {P(IPAAm-co-CIPAAm)} grafted surface accelerates the cell detachment process because of the presence of hydrophilic carboxyl group (Ebara *et al*, 2003).

7. Biomolecule immobilizing thermoresponsive substrates

Biomolecule immobilizing thermoresponsive cell culture dishes are known to be the next generation thermoresponsive substrates in cell sheet engineering (Jun and Okano, 2010). Regulation of cellular metabolisms and functions in culture are important aspects in cell sheet engineering. The simple approach to enhance the cellular activities are by immobilization or coating of bioactive compounds such as collagen, fibrin, elastin and arginine-glycine-aspartic acid (RGD) containing peptides on the cell culture substrate (Ebara *et al*, 2004). Anchoring of these bioactive compounds to this thermoresponsive substrate through chemical bonds is expected to increase the stability of the modified surface to a prolonged period. The interactions of these biomolecule ligands and cell membrane receptors have an important role in cell spreading, proliferation, differentiation, and signal transduction. This can be achieved either through coating or by the use of coupling agents or else by copolymerizing with an analogue of Isopropylacrylamide.

Cell adherence and growth on the thermoresponsive substrate is improved by coating cell adhesion promoters such as collagen, laminin and fibrin (Moran *et al*, 2007). But repeated washing will remove the coating of biomolecules. Immobilization is the process which helps to increase the stability and make possible their repeated or continued use.

Copolymerizing with an analogue of isopropylacrylamide, 2-carboxyisopropylacrylamide (CIPAAm) which has a carboxylate side chain that favours the immobilization of bioactive

molecules onto thermoresponsive substrates. The synthetic cell adhesive peptide Arg-Gly-Asp (RGD) found in extracellular matrix proteins has been successfully immobilized onto the grafted polymer chains via the above mentioned method using the coupling agents 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride [EDC]. The cells adhered and spread well on these surface even in the absence of serum. Another method of immobilization is by the coupling of biotinylated biomolecules onto streptavidin immobilized thermoresponsive substrate by affinity binding without any coupling agents. The co-immobilisation of cell adhesive peptide RGDs and cell growth factor insulin facilitated the cell adhesion and cell proliferation respectively.

The epoxy ring opening of GMA moiety is well known for incorporating diverse type of biomolecules. The uniqueness of the thermoresponsive substrate such as N-isopropylacrylamide-co-glycidylmethacrylate is that, it facilitates the immobilization of biomolecules without any coupling agents by the presence of unreacted epoxy rings.

The biomolecule immobilized surfaces facilitates cell adhesion, culture and harvest of cell sheets which enables serum free cell culture and enzyme free cell harvesting.

8. Supports for the transfer of cell sheet

A cell sheet carrier is a support which facilitates the transfer of retrieved cell sheets. An ideal material as a cell sheet carrier should possess the features like biocompatibility, surface pliability, mechanical strength, hydrophilicity, presence of surface reactive groups which enable easy modification etc. Polyvinylidene fluoride membranes in the form of annular ring, fibrin gel coated membranes are some of the systems which have been used till today. Polyion complex gels are further developed as cell sheet carriers (Tang *et al*, 2007) but possess limited mechanical strength and have application difficulties. A further innovative development in this field is the cell culture substrate with combined properties of thermoresponsiveness and cell sheet carrier tool. Modified overhead projection transparency sheet based on polyethyleneterephthalate coated with thermoresponsive N-isopropylacrylamide-Co-glycidylmethacrylate is reported to be an efficient cell culture substrate as well as a cell sheet carrier tool (Joseph *et al*, 2010). The study demonstrated that a simple modification of commercially available PET sheets by hydrolysis followed by coating of thermoresponsive NGMA forms a biocompatible substrate for scaffold free cell sheets as well as a cell sheet carrier tool.

9. Clinical applications of cell sheet engineering

Cell sheet engineering favours the formation of scaffold free transplantable three dimensional tissues from thermoresponsive cell culture substrate. This technology has been used in corneal surface reconstruction, myocardial tissue reconstruction, regeneration of damaged periodontal tissue, treatment of oesophageal ulceration, treatment for type 1 diabetes etc (Elloumi-Hannachi *et al*, 2010). Some of the clinical applications reported till dates are reported below. This technology in corneal surface reconstruction has been successfully applied to the patients suffering from unilateral or bilateral corneal stem cell deficiencies due to alkali burns or Stevens Johnson syndrome. These reconstructed corneal surfaces are able to restore the vision. Cell sheet technology for the treatment of oesophageal ulceration enhances the wound healing and reduces the host inflammatory responses. This is reported to be the first clinical case in the world which is applied to endoscopy with

successful wound healing and improved post operative quality of life. Transplantation using cell sheets derived from periodontal ligament is reported to be useful for the regeneration of damaged periodontal tissue. Cell sheet technology using pancreatic islets has been used for the treatment of type1 diabetes. Another clinical application of cell sheet engineering is its use in the treatment for dilated cardiomyopathy and cardiovascular tissue repair by myocardial tissue reconstruction. This smart culture surfaces have already been used in clinical trials and thus it offers a new frontier in the biomedical field.

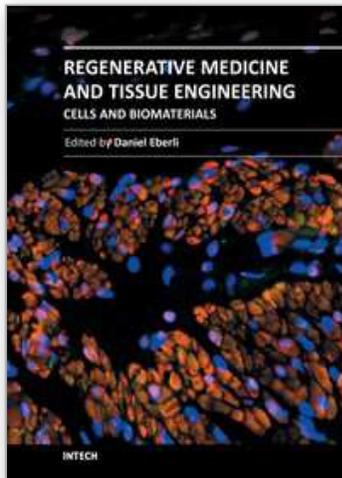
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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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