

Smart thermoresponsive coatings and surfaces for tissue engineering: switching cell-material boundaries

Ricardo M.P. da Silva^{1,2}, João F. Mano^{1,2} and Rui L. Reis^{1,2}

¹ 3Bs Research Group – Biomaterials, Biodegradables, Biomimetics, Department of Polymer Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

² Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

The smart thermoresponsive coatings and surfaces that have been explicitly designed for cell culture are mostly based on poly(*N*-isopropylacrylamide) (PNIPAAm). This polymer is characterized by a sudden precipitation on heating, switching from a hydrophilic to a hydrophobic state. Mammalian cells cultured on such thermoresponsive substrates can be recovered as confluent cell sheets, while keeping the newly deposited extracellular matrix intact, simply by lowering the temperature and thereby avoiding the use of deleterious proteases. Thermoresponsive materials and surfaces are powerful tools for creating tissue-like constructs that imitate native tissue geometry and mimic its spatial cellular organization. Here we review and compare the most representative methods of producing thermoresponsive substrates for cell sheet engineering.

Tissue culture on thermoresponsive substrates

Thermoresponsive substrates designed for tissue engineering have mainly used poly(*N*-isopropylacrylamide) (PNIPAAm) as the molecular switch for cell adhesion (ON) and detachment (OFF). The first published paper reporting the inverse solubility upon heating of PNIPAAm dates from 1967 [1]. The cloud point (at around 32°C in pure water [1–3]) is also termed inverse temperature solubility or, more generally, the lower critical solution temperature (LCST). In an aqueous environment, isolated individual PNIPAAm chains undergo a reversible conformational transition from expanded coil to compact globule as the temperature is raised above the LCST [2,3]. The solubility is affected because the amphiphilic PNIPAA chains hide the hydrophilic amide groups and expose the hydrophobic isopropyl groups in the compact globule conformation. The chemical mechanism and thermodynamic details of this phenomenon have been reviewed elsewhere [4,5].

In their natural environment, many mammalian cells do not exist in solution but interact with immobilized components of the extracellular matrix (ECM) for the development, organization and maintenance of the tissues. Consequently, anchorage-dependent cells need to adhere to solid substrates for normal functioning. Attached cells proliferate and produce ECM, forming confluent cell mono-

layers. In conventional cell culture dishes, which consist of tissue culture polystyrene (TCPS), cells are harvested by disaggregating the ECM through the proteolytic action of trypsin and by chelating the Ca²⁺ and Mg²⁺ ions with ethylenediaminetetraacetic acid (EDTA). However, non-specific proteases can damage crucial cell surface proteins, and this constitutes a major drawback of this cell harvesting method [6–10]. Moreover, from a tissue engineering perspective, the disruption of the newly formed tissue-like structures seems to be a backward step.

Thermoresponsive substrates can be created so that cells adhere and proliferate at the culture temperature, and then release the cultured cell sheets on command, by cooling below the LCST. These cell sheet engineering tools have been classified according to two distinct strategies that were demonstrated in the pioneering papers: thermoresponsive insoluble surface grafts [6] and soluble coatings [11].

The use of temperature to detach confluent cell sheets in culture, without the use of conventional enzymatic treatments, was first reported in 1990 by Takezawa *et al.* [11]. In this study, fibroblast monolayers were released at lowered temperature by dissolution of the dish coating, which consisted of a physical blend of collagen and PNIPAAm. In this method, cell sheet recovery is accomplished by disintegration of the coating.

Around the same time, Yamada *et al.* [6] reported that bovine hepatocytes, which are highly sensitive to enzymatic harvesting, could be successively subcultured on TCPS surfaces that were covalently grafted with PNIPAAm. The rationale is that the slightly hydrophobic surfaces will support cell adhesion, whereas hydrated hydrophilic surfaces will not. At the culture temperature (37°C) insoluble grafted molecules expose their hydrophobic groups, whereas below the LCST the chains become hydrophilic and hydrated.

Here we review the thermoresponsive substrates underlying several outstanding achievements in regenerative medicine. Further sophistication of such substrates will be made possible by greater understanding of the underlying principles that govern their applicability.

Insoluble PNIPAAm surfaces: fabrication methods

A large range of different methods can be used to fabricate insoluble thermoresponsive surfaces [6,9,10,12–15].

Corresponding author: da Silva, R.M.P. (ricardosilva@dep.uminho.pt).

Nevertheless, only a few of these have been found to be suitable for cell sheet engineering. Some surfaces based on PNIPAAm do not support cell adhesion even above the LCST [16,17], making them unsuitable as culture substrates. The surface fabrication methods reviewed here are limited to those that have consistently demonstrated an ability to grow cultured cells to confluence.

Electron-beam (e-beam) polymerization of PNIPAAm onto TCPS is by far the most employed method for producing thermoresponsive surfaces for cell sheet engineering [6–8,16,18–29]. Temperature-controlled cell adhesion and detachment is achieved for grafting densities in the range of 1.4–2 µg/cm² (thickness 15–20 nm) [16,18–22]. Grafted PNIPAAm layers thicker than 30 nm (2.9–3 µg/cm²) do not support cell adhesion at any temperature [16,17].

A second type of thermoresponsive surface that is covalently attached to a solid substrate is produced by plasma polymerization of PNIPAAm [10,30]. Plasma glow discharge power is gradually decreased to form an adhesion-promoting layer on the substrate and to deposit a functional coating at the outer surface. Plasma-deposited PNIPAAm was found to be consistent with a crosslinked structure, which largely retained the monomeric structure and preserved the PNIPAAm phase transition [31,32]. The sum frequency generation (SFG) vibrational spectra obtained for this type of graft above the LCST suggested that the hydrophobic isopropyl side groups were oriented towards the aqueous environment. By contrast, the spectra obtained at room temperature provided evidence for a disordering of the isopropyl groups away from the surface normal (the perpendicular). Furthermore, the characteristic peaks of amide groups were not detected; this suggests that the previous hypothesis, that these groups are exposed to the aqueous environment to participate in hydrogen bonding below the LCST, is possibly not correct [32]. Finally, cell culture studies and thermal lift-off did not show obvious differences between different batches of plasma-deposited coatings, although the thickness of deposited polymer varied from batch to batch [10,30]. This shows that cell adhesion and proliferation do not seem to be sensitive to the grafted layer thickness of plasma-polymerized PNIPAAm substrates.

Another method of producing thermoresponsive surfaces involves the partial entrapment of a copolymer of PNIPAAm and 4-(N-cinnamoylcarbamide)methylstyrene (CCMS) onto TCPS and irradiation with ultraviolet (UV) light to crosslink the copolymer through the dimerization of the cinnamoyl groups [9,33]. Similarly to plasma-polymerized PNIPAAm, cell adhesion and proliferation were

not sensitive to the thickness of the functional layer in the range 2.4–6.9 µg/cm² [33].

Another approach to functionalizing TCPS surfaces makes use of a PNIPAAm copolymer derivatized with photo-reactive 4-azidoaniline groups [34–37]. UV light irradiation produces a heavily crosslinked surface layer [38]. This method has been employed to produce micropatterned surfaces with thermoresponsive regions by photolithography in which the solvent cast coating is washed out from shadowed regions [34–37]. Using this approach, fibroblasts were cultured for short periods (2 h) and adhered to the micropatterned substrates. Afterwards, the cells were selectively detached from the grafted regions [34,35]. This technique demonstrated that spatial control over detachment of adhered cells can be achieved, and might be a useful tool to control the distribution of different cell types in coculture systems.

All the methods described in this section use the hydrophobic-to-hydrophilic switch to recover cell sheets, instead of disintegration of the coating. This ensures that the coating is not harvested with the cell sheet, keeping the cell construct free of unwanted soluble polymer after detachment.

Insoluble PNIPAAm surfaces: achieving cell adhesion and proliferation

Although all thermoresponsive substrates based on PNIPAAm showed the expected hydrophilic properties below the LCST (Tables 1 and 2), only some were able to support cell adhesion and proliferation above the LCST; in others, cells did not form contiguous monolayers. For instance, the surfaces described above as consisting of thin layers of crosslinked PNIPAAm immobilized on solid supports, do support cell adhesion and proliferation. By contrast, cells fail to adhere on e-beam-grafted PNIPAAm surfaces thicker than 30 nm [16,17], or on PNIPAAm crosslinked with methylenebis(acrylamide) (MBAAm) [16,17] or non-crosslinked PNIPAAm homopolymer [11]. Cell adhesion can be correlated to the adsorption of adhesive proteins, such as fibronectin. The e-beam-grafted PNIPAAm at the greatest density and the bulk crosslinked hydrogel fail to adsorb fibronectin [16,17]. Understanding why only certain molecular architectures produce surfaces with appropriate properties could facilitate the engineering of more sophisticated thermoresponsive surfaces with increased functionalities.

The equilibrium water contact angle (measured under static conditions) of some polymer substrates has been shown to correlate with cell adhesion and proliferation,

Table 1. Temperature-induced surface free energy changes as sensed by the static water contact angle measured above and below the LCST

Surface description	T > LCST	T < LCST	Cell adhesion	Refs
Bulk PNIPAAm hydrogel crosslinked with MBAAm (1.3%)	49.4° (T = 40°C) ^a	11.5° (T = 10°C) ^a	Nonadhesive	[16]
e-Beam polymerized PNIPAAm (1.4 µg/cm ²)	77.9° (T = 37°C) ^a	65.2° (T = 20°C) ^a	OK	[16]
e-Beam polymerized PNIPAAm (2.9 µg/cm ²)	69.5° (T = 37°C) ^a	60.0° (T = 20°C) ^a	Nonadhesive	[16]
e-Beam polymerized PNIPAAm (1.6 µg/cm ²)	66° (T = 37°C) ^b	54° (T = 20°C) ^b	OK	[41]
Plasma polymerized PNIPAAm (1 W)	40° (T = 40°C) ^a	34° (T = 20°C) ^a	OK	[32]
Plasma polymerized PNIPAAm (5 W, 55°C)	42.5° (T = 45°C) ^a	33.5° (T = 20°C) ^a	NA	[40]

^aCaptive air bubble method.

^bSessile drop method.

NA, cell adhesion studies were not reported. OK, cells adhere, proliferate and detach as a confluent sheet.

Table 2. Temperature-induced surface free energy changes associated with the dispersive component as sensed by the advancing water contact angle measured above and below the LCST

Surface description	$T > LCST$	$T < LCST$	Cell adhesion	Refs
UV crosslinked P(NIPAAm-co-CCMS) (0.7%)	87.8° ($T = 37^\circ C$) ^a	37.2° ($T = 19^\circ C$) ^a	OK	[33]
UV crosslinked P(NIPAAm-co-CCMS) (1.2%)	92.6° ($T = 37^\circ C$) ^a	40.5° ($T = 19^\circ C$) ^a	OK	[33]
Bulk PNIPAAm hydrogel crosslinked with MBAAm (1%)	90° ($T = 36^\circ C$) ^b	42° ($T = 25^\circ C$) ^b	NA	[64]
Bulk PNIPAAm hydrogel crosslinked with MBAAm (1.8%)	90° ($T = 36^\circ C$) ^b	42° ($T = 25^\circ C$) ^b	NA	[65]
Bulk PNIPAAm hydrogel crosslinked with MBAAm (2.2%)	60° ($T = 37^\circ C$) ^{a,c}	25° ($T = 20^\circ C$) ^{a,c}	NA	[66]
Bulk PNIPAAm hydrogel crosslinked with MBAAm (10%)	90° ($T = 37^\circ C$) ^{a,c}	30° ($T = 20^\circ C$) ^{a,c}	NA	[66]

^aDynamic Wilhelmy plate method.^bSessile drop method.^cEstimated from a chart.

NA, cell adhesion studies were not reported in these articles, but it has been reported that cells do not adhere on bulk PNIPAAm hydrogel crosslinked with MBAAm [16,17]. OK, cells adhere, proliferate and detach as a confluent sheet.

which are both optimal at an angle of around 70° [39]. Using this correlation, the unsuitability of bulk crosslinked PNIPAAm hydrogels for supporting cell adhesion can be explained by their greatly hydrophilic nature, which is revealed by a low static water contact angle, even above the LCST (see Table 1) [16]. However, plasma-deposited PNIPAAm, which has been shown to support reversible cell adhesion and detachment [32], gave similar values for the static water contact angle [32,40]. Also, the dependence of cell adhesion on the thickness of PNIPAAm grafted onto TCPS by e-beam irradiation cannot be clearly correlated with the differences found in the static water contact angle, because the reported values (Table 1) are close to what is regarded as optimal for cell adhesion. Besides, as the values in Table 1 show, the nonadhesive surface with the greatest grafting density (2.9 µg/cm²) showed a contact angle between the values found for lower densities (1.4 and 1.6 µg/cm²), which are in turn appropriate for cell adhesion and proliferation [16,41]. These findings suggest that an additional effect other than the surface wettability, but nevertheless strongly related to the grafting density or thickness, is responsible for inhibiting protein adsorption onto e-beam-grafted surfaces at the greatest density and, consequently, hindering cell adhesion.

The performance of the surfaces can also be affected by the swelling ability. Akiyama *et al.* reported that swelling was significantly reduced for crosslinked gels immobilized on a rigid surface and that the swelling ratio was further lowered for the thinner crosslinked gels. Consequently, they suggested that the surface immobilization of the crosslinked chains dramatically restricted the molecular motion, and extrapolated that those restrictions on molecular mobility would be more significant in the vicinity of the rigid support surface, as in the case of the e-beam-grafted PNIPAAm on TCPS [16], enabling cell adhesion below a suitable thickness limit.

It is well established that, under suitable grafting densities and sufficient molecular mobility [42–44], immobilized nonionic and greatly hydrated macromolecules such as polyethylene glycol (PEG) can prevent (or minimize) protein adsorption and, consequently, render surfaces resistant to cell adhesion. The nonfouling nature of those surfaces is often interpreted in terms of the ‘steric repulsion’ model. It is considered that the solvation of the PEG chains together with their great conformational freedom and hence entropy prevents the protein adsorption that, otherwise, would restrict the conformational mobility

of the hydrated free polymer molecules [42,45,46]. The nonfouling property of PEG chains has been used to explain the nonadhesive property of hydrated PNIPAAm surfaces below the LCST [15,17,47].

However, the same ‘steric repulsion’ model is less successful at explaining the nonfouling nature of the thicker e-beam PNIPAAm-grafted surfaces (2.9 µg/cm²) above the LCST. In fact, during the volume transition it is expected that the PNIPAAm molecules dehydrate, at least partially, and expose their hydrophobic isopropyl groups, as was reported for plasma-polymerized NIPAAm [32]. In Figure 1, crosslinked PNIPAAm chains are schematically represented above the LCST. The isopropyl groups are oriented towards the interface with the water, and the dependence of protein adsorption on the grafting thickness and chain mobility is illustrated. The decrease of the PNIPAAm chain density with increasing grafting thickness is based on the model proposed by Kikuchi and Okano [48]. Similarly, surfaces containing methoxy-terminated oligo(ethylene glycol) self-assembled monolayers (SAMs) on gold resist protein adsorption, despite the hydrophobic group pointing to the water interface [43,46]. By contrast, protein adsorption is detected for the same SAMs on silver, which form a denser SAM phase, thereby restricting access to water molecules and molecular mobility of the oligo(ethylene glycol) tails [46]. The ‘steric repulsion’ model does not explain the nonfouling nature of some short oligomers, but this nature can be interpreted based on the ‘water barrier’ theory, that is, protein cannot adsorb because of a tightly bound water layer [42,46]. The similarity between the results on the methoxy-terminated oligo(ethylene glycol)-SAMs and the dependence of protein adsorption on the thickness of the e-beam-grafted PNIPAAm layer, and its consequences for cell adhesion behaviour (see Figure 1), is remarkable. Greater restrictions to the molecular mobility and lower hydration, which have been associated with the lower thicknesses of e-beam-grafted PNIPAAm, are in turn correlated with the denser molecular packing of SAMs on the model surfaces.

The dynamic contact angle, that is, the measurement of the ‘advancing’ and ‘receding’ contact angles at the moving three phase boundary (solid–liquid–air), was also used to study the properties of insoluble PNIPAAm surfaces. Johnson and Dettre proposed a model for dynamic contact angle hysteresis caused by surface chemical heterogeneity; they predicted that the advancing contact

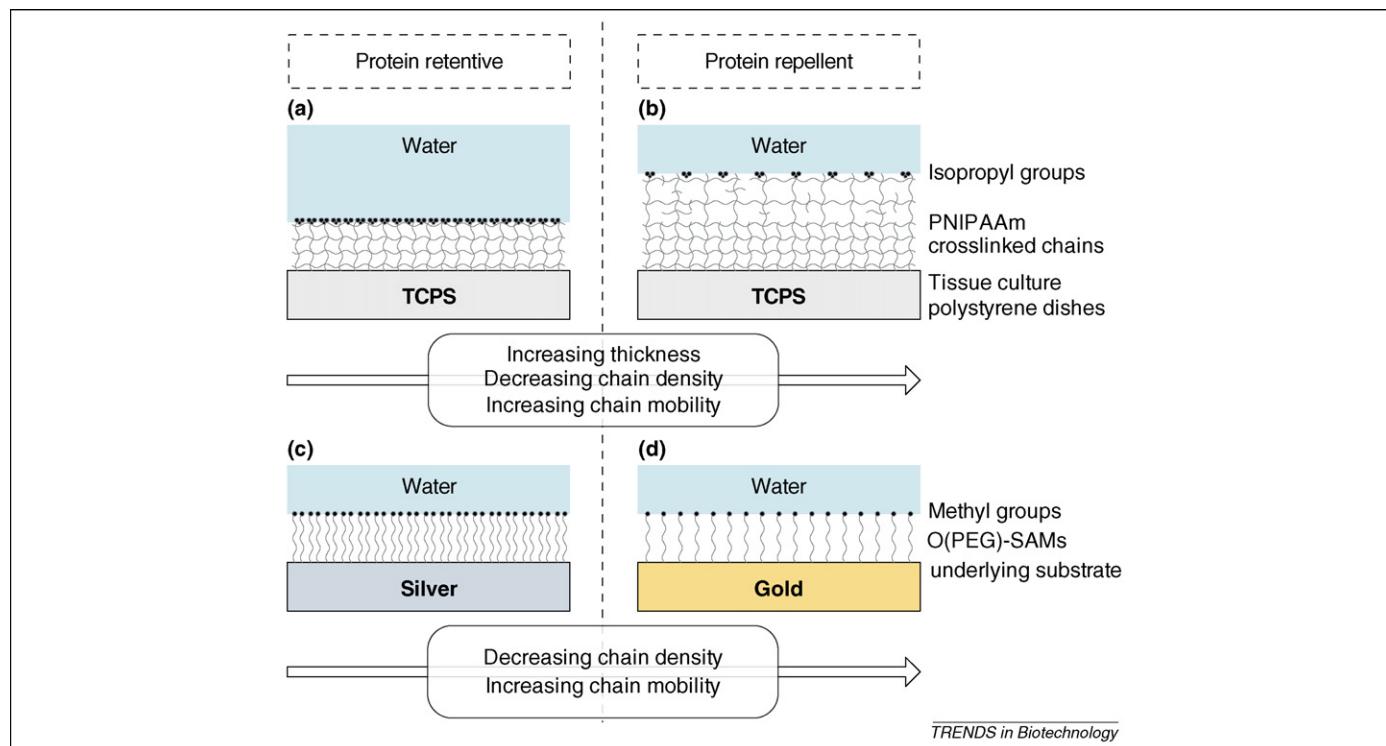


Figure 1. (a) Schematic representation of the e-beam-grafted insoluble PNIPAAm surfaces. At culture temperatures above the LCST, the isopropyl groups point to the interface with the water, conferring hydrophobic properties on the surface. At low graft thicknesses, adhesive proteins will adsorb and cells can adhere to the surface. (b) As the graft thickness increases, the PNIPAAm chain density decreases (lower surface concentration of isopropyl groups) and the surface becomes unsuitable for cell culture. (c) Methoxy-terminated oligo(ethylene glycol) self-assembled monolayers (SAMs) behave in a similar way to (a) and (b). These SAMs expose the hydrophobic methyl groups to the water interface. The SAMs on silver form greatly compacted structures that were found to adsorb proteins. (d) The same molecules form less compacted SAMs on gold producing nonfouling properties typical of other oligo(ethylene glycol) molecules. The black dots represent the hydrophobic isopropyl and methyl groups of PNIPAAm and O(PEG)-SAMs, respectively. (a) and (b) adapted, with permission, from reference [48].

angle would be associated with the surface concentration of hydrophobic regions [49,50]. They also found that the advancing contact angle does not vary greatly for elevated concentrations of the hydrophobic regions, but decreases sharply when the concentration of the latter is reduced to a low coverage ratio [49,50]. When analysing the values of the advancing water contact angle reported by several authors (Table 2), and considering the nonadhesive nature of bulk PNIPAAm hydrogels crosslinked with MBAAm, no clear tendency can be seen correlating these values with the suitability of the surface to support cell growth. However, similar elevated values for advancing contact angle of the surfaces above the LCST confirm the raised concentration of hydrophobic regions, but can still hide considerable differences in the concentration of isopropyl groups. Moreover, a low advancing contact angle (Table 2) below the LCST should mean that most of the isopropyl groups are no longer oriented to the interface. This is corroborated by SFG vibrational spectra, which provide evidence for the orientation of the isopropyl groups towards the aqueous environment above the LCST, and their disordering away from the surface normal at lower temperatures [32].

In conclusion, cell adhesion and proliferation on insoluble PNIPAAm surfaces cannot be described only in terms of surface wettability, because above the LCST the surfaces are only partially dehydrated. Interdependent variables such as swelling ratio, molecular mobility, chain density and concentration of the hydrophobic groups must be simultaneously addressed.

Insoluble PNIPAAm surfaces: mechanisms underlying cell sheet detachment

Spontaneous cell sheet detachment is driven by physical events, such as an increased hydrophilicity, that are induced by lowering the temperature below the LCST. Nevertheless, detachment seems also to be mediated by active cellular metabolic processes, given that cell detachment can be suppressed by adding an ATP synthesis inhibitor [51]. Selective inhibition of actin filaments, by means of either an actin stabilizer or an actin depolymerizer, strongly indicates that forces exerted by the cytoskeleton are important in the detachment process [51]. Moreover, cells of different phenotypes require varying optimum temperatures for detachment, depending on their differing temperature sensitivities for cellular metabolic processes [52]. Furthermore, each specific cell phenotype creates different ECM structures, and it is expected that deposited basal structures could also be different. This could also have implications for the response to the thermoresponsive switch of cell sheets created from different cell sources. For example, fibronectin, which was deposited as a matrix composed of thin fibrils on the thermoresponsive surface, could be recovered together with the confluent monolayer of bovine aortic endothelial cells by low-temperature lift-off [53]. This is in good agreement with the resistance exhibited by e-beam PNIPAAm-grafted surfaces to adsorb the soluble form of fibronectin below the LCST [16]. In addition, bovine aortic endothelial cell sheets grown on plasma-polymerized NIPAAm surfaces left behind little (if any) laminin or fibronectin after

thermal harvesting. However, a detailed analysis of the thermoresponsive surface after cell sheet detachment showed evidence of a protein film consistent with collagen types I and IV [10,30], indicating that a fraction of some of the ECM proteins that cells deposit in the culture surfaces remains in that substrate after thermal lift-off. Canavan *et al.* suggested that, because cells mainly interact with the ECM at focal points, the ECM proteins that do not have strong interactions with cells could be retained in the substrate after thermal recovery of the cell sheet [10]. Finally, it is also reasonable to consider that thermoresponsive surfaces below the LCST have a different adhesiveness towards ECM proteins. Cell sheets could resist detachment because of proteins that function as anchoring points and therefore remain attached to the surface after detachment. This might explain why cell sheets do not detach when the cytoskeletal contractile forces are selectively inhibited.

Cell sheet engineering and manipulation

When confluent cell sheets are harvested from thermoresponsive dishes, the cellular contractile forces exerted by the cytoskeleton are no longer supported by the forces of adhesion to the solid surface. As a result, the cell sheet starts to shrink [20] and to aggregate resulting in multicellular spheroids [11]. Hydrophilic membranes placed on top of the cell sheets adhere to their apical surface upon removing the culture medium, possibly because of capillary forces. When the temperature is lowered, the cell sheet is released but still stuck to the supporting membranes, which provide a physical support for the manipulation of the delicate cell sheets and enable their transfer from the thermoresponsive surface to the desired substrate [18,20–22,24,27]. The basal surface of the cell sheet will adhere to the new substrate, possibly through the remaining adhesive proteins that were present at the interface with the previous thermoresponsive surface and harvested with the cell sheet [53]. In a final step, the supporting membrane will spontaneously detach by adding culture medium. Cell sheet manipulation techniques have been established notably by the group led by Dr T. Okano; after their pioneering paper [6], this team systematically investigated insoluble PNIPAAm-grafted substrates to produce different cell sheets from several cell phenotypes [48,54,55].

The advantages of cell sheet engineering over more conventional tissue engineering strategies are described in Box 1. Single cell sheets have been produced for regeneration of cornea [7,23,28,29] and epidermis [18]. When directly transplanted to human patients for corneal regeneration, cell sheets adhered rapidly, avoiding the need for sutures [29]. In comparison, conventional corneal and epidermal tissue engineering approaches use dispase to harvest corneal epithelial cell sheets and multilayered keratinocyte sheets from the culture dishes. These non-specific proteases damage the formed ECM and other proteins, leading to fragility of the cell sheets and disrupting differentiated structures and functions [7,18,19]. Residual dispases can also be harmful to the transplantation site and jeopardize the treatment.

An alternative tissue engineering approach makes use of biodegradable scaffolds, such as collagen [56]. However,

Box 1. Advantages of cell sheet engineering compared with more conventional approaches

- Cell sheets harvested from thermoresponsive substrates adhere to the host tissues without the need for sutures [29].
- Tissue engineering strategies require the scaffold material to be surgically placed between cultured cells and the injury site, which can complicate or delay integration of the tissue implant [55].
- Cell sheet engineering avoids the use of scaffold materials, which can have potential for inflammatory or foreign body reactions or other complications arising from the by-products of scaffold biodegradation.
- Thermoresponsive substrates avoid the use of deleterious enzymes that typically are used to remove cell monolayers from conventional culture dishes [7,18,19].
- Control over the spatial distribution of cells within three-dimensional stratified tissues can be achieved by layering cell sheets created from different cell types [21].
- Cell sheet engineering offers better control over cell seeding; that is, when cells are seeded at a cell concentration similar to that observed on a confluent monolayer, final tissue-like constructs have greater cell densities and less ECM, reflecting the varying cell densities of the different native tissues [20,25].

this technique requires that scaffold material is surgically placed between the cultured cells and the host tissue at the injured (or diseased) site. Unless the use of scaffolds is crucial for treatment, such as to provide short-term mechanical support, cell sheet engineering can be an appealing alternative. For example, if a scaffold is used only to recover and manipulate cultured tissue, replacing it with engineered cell sheets has great advantages – in avoiding a possible inflammatory or foreign body reaction or other complications arising from the by-products of scaffold biodegradation.

Moreover, from the tissue organization point of view, it might be desirable to avoid the use of scaffolds, whose supramolecular organization does not match that of the ECM deposited by the cells. For instance, in native cardiac muscle, cells are densely packed with diffuse gap junctions, important for electrical transmission. Layered cardiomyocyte cell sheets showed a greater cell density with diffuse gap junctions and less ECM deposition, than constructs that were created using biodegradable scaffolds [20,25]. Further, cell sheet engineered cardiac grafts showed synchronized pulsation [25].

This method was also used to create cell sheets of hepatocytes and endothelial cells that were subsequently layered to mimic the three-dimensional cellular organization in liver lobules. It was found that the hepatocytes cocultured in the layered system maintained differentiated functions for over 41 days, whereas these functions disappeared within 10 days in hepatocyte monoculture controls [21].

It should be emphasized that in all the examples given above of applications for cell sheet engineering, the cell sheets were obtained using insoluble thermoresponsive surfaces. In fact, the majority of the cell sheet engineering applications were developed for insoluble thermoresponsive surfaces. It was only recently that the attention of cell sheet engineers turned again toward the approach based on thermoresponsive substrates that become soluble below the LCST. Endothelial cells were cultured until confluence

on coatings of PNIPAAm-grafted gelatin produced by living radical photopolymerization [57–63]. Gelatin, the denatured form of collagen, was used to address the nonadhesive nature of soluble PNIPAAm. These cells were shown to detach successfully when the temperature was lowered to 20 °C [58]. Moreover, a tubular monolayer of endothelial cells was created by coating a glass capillary tube with the same PNIPAAm-grafted gelatin. An engineered vascular graft could be recovered that consisted of the endothelial cells and secreted ECM. The resulting tubular endothelium resembled the intimal layer of a native blood vessel [57].

The disintegration of the coating could be a potential drawback of this method, because it poses the risk that the harvested cell sheet will be contaminated with soluble polymer after detachment. Nevertheless, the method gives the cell sheet engineer the ability to release biological structures from intricate supportive moulds, enabling the design and one-step fabrication of ‘cell sheets’ with complex geometries resembling the native tissue shapes.

Concluding remarks

Thermoresponsive surfaces that consist of thin layers of crosslinked PNIPAAm on a nonthermoresponsive solid support were initially proposed as new technologies for cell proliferation. However, since then thermoresponsive surfaces have been developed into valuable tools for engineering cell sheets and used successfully to produce tissue-like structures with interesting and important applications in regenerative medicine. So far, this technology is being applied for the regeneration of cornea [7,23,28,29], skin [18], liver [21], heart [20,25] and blood vessels [57].

The insoluble PNIPAAm surfaces are suitable for producing cell sheets that seem to be ideal for applications where the native tissues show stratified structures. Cell sheets can be layered to obtain thicker tissue-like constructs composed of single [25] or multiple cell phenotypes [21]. Moreover, the low-temperature soluble coatings that can support cell adhesion and proliferation above the LCST have great potential for engineering ‘cell sheets’ with more complex geometries resembling tissue shapes, which can only be obtained after complete removal of supporting materials.

Despite the advances that thermoresponsive coatings and surfaces have already made for the regeneration of injured tissues, cell sheet engineering is still in its infancy and has enormous research potential. The scarcity of suitable cell sources is an important obstacle to developing clinically relevant applications, a common issue in the field of regenerative medicine. Future developments in stem cell technologies combined with a full toolbox of thermoresponsive technologies promise great benefits for human health and welfare.

Acknowledgements

This work was partially supported by the Portuguese Foundation for Science and Technology (FCT), funds from the POCTI and FEDER programmes, the scholarship SFRH/BD/6862/2001 granted to R.M.P.S. and the project PTDC/QUI/68804/2006. This work was carried out under the scope of the European NoE EXPERTISSUES (NMP3-CT-2004-500283).

References

- Scarpa, J.S. *et al.* (1967) Slow hydrogen-deuterium exchange in a non-alfa-helical polyamide. *J. Am. Chem. Soc.* 89, 6024–6030
- Fujishige, S. *et al.* (1989) Phase-transition of aqueous-solutions of poly(*N*-isopropylacrylamide) and poly(*N*-isopropylmethacrylamide). *J. Phys. Chem.* 93, 3311–3313
- Kubota, K. *et al.* (1990) Single-chain transition of poly(*N*-isopropylacrylamide) in water. *J. Phys. Chem.* 94, 5154–5158
- Graziano, G. (2000) On the temperature-induced coil to globule transition of poly-*N*-isopropylacrylamide in dilute aqueous solutions. *Int. J. Biol. Macromol.* 27, 89–97
- Baysal, B.M. and Karasz, F.E. (2003) Coil-globule collapse in flexible macromolecules. *Macromol. Theor. Simul.* 12, 627–646
- Yamada, N. *et al.* (1990) Thermoresponsive polymeric surfaces – control of attachment and detachment of cultured-cells. *Makromol. Chem., Rapid Commun.* 11, 571–576
- Ide, T. *et al.* (2006) Structural characterization of bioengineered human corneal endothelial cell sheets fabricated on temperature-responsive culture dishes. *Biomaterials* 27, 607–614
- Nakajima, K. *et al.* (2001) Intact microglia are cultured and non-invasively harvested without pathological activation using a novel cultured cell recovery method. *Biomaterials* 22, 1213–1223
- Von Recum, H.A. *et al.* (1999) Maintenance of retinoid metabolism in human retinal pigment epithelium cell culture. *Exp. Eye Res.* 69, 97–107
- Canavan, H.E. *et al.* (2005) Cell sheet detachment affects the extracellular matrix: a surface science study comparing thermal lift-off, enzymatic, and mechanical methods. *J. Biomed. Mater. Res. A* 75, 1–13
- Takezawa, T. *et al.* (1990) Cell-culture on a thermoresponsive polymer surface. *Biol. Technol.* 8, 854–856
- Ista, L.K. *et al.* (2001) Synthesis of poly(*N*-isopropylacrylamide) on initiator-modified self-assembled monolayers. *Langmuir* 17, 2552–2555
- Taniguchi, T. *et al.* (2003) Adsorption/desorption behavior and covalent grafting of an antibody onto cationic amino-functionalized poly(styrene-*N*-isopropylacrylamide) core-shell latex particles. *Colloids Surf. B Biointerfaces* 29, 53–65
- Sakamoto, C. *et al.* (2004) Temperature- and pH-responsive aminopropyl-silica ion-exchange columns grafted with copolymers of *N*-isopropylacrylamide. *J. Chromatogr.* 1030, 247–253
- Huber, D.L. *et al.* (2003) Programmed adsorption and release of proteins in a microfluidic device. *Science* 301, 352–354
- Akiyama, Y. *et al.* (2004) Ultrathin poly(*N*-isopropylacrylamide) grafted layer on polystyrene surfaces for cell adhesion/detachment control. *Langmuir* 20, 5506–5511
- Yamato, M. *et al.* (2003) Nanofabrication for micropatterned cell arrays by combining electron beam-irradiated polymer grafting and localized laser ablation. *J. Biomed. Mater. Res. A* 67, 1065–1071
- Yamato, M. *et al.* (2001) Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. *Tissue Eng.* 7, 473–480
- Shiroyanagi, Y. *et al.* (2003) Transplantable urothelial cell sheets harvested noninvasively from temperature-responsive culture surfaces by reducing temperature. *Tissue Eng.* 9, 1005–1012
- Shimizu, T. *et al.* (2001) Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. *Tissue Eng.* 7, 141–151
- Harimoto, M. *et al.* (2002) Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. *J. Biomed. Mater. Res.* 62, 464–470
- Nandkumar, M.A. *et al.* (2002) Two-dimensional cell sheet manipulation of heterotypically co-cultured lung cells utilizing temperature-responsive culture dishes results in long-term maintenance of differentiated epithelial cell functions. *Biomaterials* 23, 1121–1130
- Sumide, T. *et al.* (2005) Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. *FASEB J.* 20, 392–394
- Shimizu, T. *et al.* (2002) Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. *J. Biomed. Mater. Res.* 60, 110–117

- 25 Shimizu, T. *et al.* (2002) Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ. Res.* 90, E40–E48
- 26 Memon, I.A. *et al.* (2005) Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets. *J. Thorac. Cardiovasc. Surg.* 130, 1333–1341
- 27 Kushida, A. *et al.* (2001) Two-dimensional manipulation of differentiated Madin-Darby canine kidney (MDCK) cell sheets: The noninvasive harvest from temperature-responsive culture dishes and transfer to other surfaces. *J. Biomed. Mater. Res.* 54, 37–46
- 28 Nishida, K. *et al.* (2004) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface. *Transplantation* 77, 379–385
- 29 Nishida, K. *et al.* (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N. Engl. J. Med.* 351, 1187–1196
- 30 Canavan, H.E. *et al.* (2005) Surface characterization of the extracellular matrix remaining after cell detachment from a thermoresponsive polymer. *Langmuir* 21, 1949–1955
- 31 Pan, Y.V. *et al.* (2001) Plasma polymerized *N*-isopropylacrylamide: Synthesis and characterization of a smart thermally responsive coating. *Biomacromolecules* 2, 32–36
- 32 Cheng, X. *et al.* (2005) Surface chemical and mechanical properties of plasma-polymerized *N*-isopropylacrylamide. *Langmuir* 21, 7833–7841
- 33 von Recum, H.A. *et al.* (1998) Novel thermally reversible hydrogel as detachable cell culture substrate. *J. Biomed. Mater. Res.* 40, 631–639
- 34 Ito, Y. *et al.* (1997) Patterned immobilization of thermoresponsive polymer. *Langmuir* 13, 2756–2759
- 35 Chen, G. *et al.* (1998) Effect of protein and cell behavior on pattern-grafted thermoresponsive polymer. *J. Biomed. Mater. Res.* 42, 38–44
- 36 Liu, H. and Ito, Y. (2002) Cell attachment and detachment on micropattern-immobilized poly(*N*-isopropylacrylamide) with gelatin. *Lab Chip* 2, 175–178
- 37 Liu, H. and Ito, Y. (2003) Gradient micropattern immobilization of a thermo-responsive polymer to investigate its effect on cell behavior. *J. Biomed. Mater. Res A* 67, 1424–1429
- 38 Sugawara, T. and Matsuda, T. (1994) Novel surface graft-copolymerization method with micron-order regional precision. *Macromolecules* 27, 7809–7814
- 39 Tamada, Y. and Ikada, Y. (1994) Fibroblast growth on polymer surfaces and biosynthesis of collagen. *J. Biomed. Mater. Res.* 28, 783–789
- 40 Bullett, N.A. *et al.* (2006) Chemical and thermo-responsive characterisation of surfaces formed by plasma polymerisation of *N*-isopropylacrylamide. *Surf. Interface. Anal.* 38, 1109–1116
- 41 Kwon, O.H. *et al.* (2000) Rapid cell sheet detachment from poly(*N*-isopropylacrylamide)-grafted porous cell culture membranes. *J. Biomed. Mater. Res.* 50, 82–89
- 42 Li, L.Y. *et al.* (2005) Protein adsorption on oligo(ethylene glycol)-terminated alkanethiolate self-assembled monolayers: the molecular basis for nonfouling behavior. *J. Phys. Chem. B* 109, 2934–2941
- 43 Prime, K.L. and Whitesides, G.M. (1993) Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide) – a model system using self-assembled monolayers. *J. Am. Chem. Soc.* 115, 10714–10721
- 44 Michel, R. *et al.* (2005) Influence of PEG architecture on protein adsorption and conformation. *Langmuir* 21, 12327–12332
- 45 Menz, B. *et al.* (2005) Impedance and QCM analysis of the protein resistance of self-assembled PEGylated alkanethiol layers on gold. *Biomaterials* 26, 4237–4243
- 46 Feldman, K. *et al.* (1999) Probing resistance to protein adsorption of oligo(ethylene glycol)-terminated self-assembled monolayers by scanning force microscopy. *J. Am. Chem. Soc.* 121, 10134–10141
- 47 Kidoaki, S. *et al.* (2001) Thermoresponsive structural change of a poly(*N*-isopropylacrylamide) graft layer measured with an atomic force microscope. *Langmuir* 17, 2402–2407
- 48 Kikuchi, A. and Okano, T. (2005) Nanostructured designs of biomedical materials: applications of cell sheet engineering to functional regenerative tissues and organs. *J. Control. Release* 101, 69–84
- 49 Johnson, R.E. and Dettre, R.H. (1964) Contact angle hysteresis. III. Study of an idealized heterogeneous surface. *J. Phys. Chem.* 68, 1744–1749
- 50 Dettre, R.H. and Johnson, R.E. (1965) Contact angle hysteresis. IV. Contact angle measurements on heterogeneous surfaces. *J. Phys. Chem.* 69, 1507–1514
- 51 Yamato, M. *et al.* (1999) Signal transduction and cytoskeletal reorganization are required for cell detachment from cell culture surfaces grafted with a temperature-responsive polymer. *J. Biomed. Mater. Res.* 44, 44–52
- 52 Okano, T. *et al.* (1995) Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. *Biomaterials* 16, 297–303
- 53 Kushida, A. *et al.* (1999) Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces. *J. Biomed. Mater. Res.* 45, 355–362
- 54 Shimizu, T. *et al.* (2003) Cell sheet engineering for myocardial tissue reconstruction. *Biomaterials* 24, 2309–2316
- 55 Yang, J. *et al.* (2005) Cell sheet engineering: Recreating tissues without biodegradable scaffolds. *Biomaterials* 26, 6415–6422
- 56 Duan, X. and Sheardown, H. (2006) Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: Mechanical properties and corneal epithelial cell interactions. *Biomaterials* 27, 4608–4617
- 57 Matsuda, T. (2004) Poly(*N*-isopropylacrylamide)-grafted gelatin as a thermoresponsive cell-adhesive, mold-releasable material for shape-engineered tissues. *J. Biomater. Sci. Polym. Ed.* 15, 947–955
- 58 Morikawa, N. and Matsuda, T. (2002) Thermoresponsive artificial extracellular matrix: *N*-isopropylacrylamide-graft-copolymerized gelatin. *J. Biomater. Sci. Polym. Ed.* 13, 167–183
- 59 Ohya, S. *et al.* (2005) Poly(*N*-isopropylacrylamide) (PNIPAM)-grafted gelatin hydrogel surfaces: interrelationship between microscopic structure and mechanical property of surface regions and cell adhesiveness. *Biomaterials* 26, 3105–3111
- 60 Ohya, S. and Matsuda, T. (2005) Poly(*N*-isopropylacrylamide) (PNIPAM)-grafted gelatin as thermoresponsive three-dimensional artificial extracellular matrix: molecular and formulation parameters vs cell proliferation potential. *J. Biomater. Sci. Polym. Ed.* 16, 809–827
- 61 Ibusuki, S. *et al.* (2003) Tissue-engineered cartilage using an injectable and *in situ* gelable thermoresponsive gelatin: fabrication and *in vitro* performance. *Tissue Eng.* 9, 371–384
- 62 Ibusuki, S. *et al.* (2003) System-engineered cartilage using poly(*N*-isopropylacrylamide)-grafted gelatin as *in situ*-formable scaffold: *in vivo* performance. *Tissue Eng.* 9, 1133–1142
- 63 Ibusuki, S. *et al.* (2003) Tissue-engineered cartilage using thermoresponsive gelatin as an *in situ* forming and moldable scaffold with chondrocytes: *in vitro* and *in vivo* performances. *Arthritis Res. Ther.* 5, S19–S20
- 64 Liang, L. *et al.* (2000) Surfaces with reversible hydrophilic/hydrophobic characteristics on cross-linked poly(*N*-isopropylacrylamide) hydrogels. *Langmuir* 16, 8016–8023
- 65 Zhang, J. *et al.* (1995) Temperature-dependent contact angles of water on poly(*N*-isopropylacrylamide) gels. *Langmuir* 11, 2301–2302
- 66 Liang, L. *et al.* (1999) Preparation of composite-crosslinked poly(*N*-isopropylacrylamide) gel layer and characteristics of reverse hydrophilic-hydrophobic surface. *J. Appl. Polym. Sci.* 72, 1–11