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Thermoresponsive polymers and their biomedical application in tissue engineering – a review

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Thermoresponsive polymers hold great potential in the biomedical field, since they enable the fabrication of cell sheets, *in situ* drug delivery and 3D-printing under physiological conditions. In this review we provide an overview of several thermoresponsive polymers and their application, with focus on poly(*N*-isopropylacrylamide)-surfaces for cell sheet engineering. Basic knowledge of important processes like protein adsorption on surfaces and cell adhesion is provided. For different thermoresponsive polymers, namely PNIPAm, Pluronics, elastin-like polypeptides (ELP) and poly(*N*-vinylcaprolactam) (PNVCL), synthesis and basic chemical and physical properties have been described and the mechanism of their thermoresponsive behavior highlighted. Fabrication methods of thermoresponsive surfaces have been discussed, focusing on PNIPAm, and describing several methods in detail. The latter part of this review is dedicated to the application of the thermoresponsive polymers and with regard to cell sheet engineering, the process of temperature-dependent cell sheet detachment is explained. We provide insight into several applications of PNIPAm surfaces in cell sheet engineering. For Pluronics, ELP and PNVCL we show their application in the field of drug delivery and tissue engineering. We conclude, that research of thermoresponsive polymers has made big progress in recent years, especially for PNIPAm since the 1990s. However, manifold research possibilities, *e.g.* in surface fabrication and 3D-printing and further translational applications are conceivable in near future.

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1. Introduction

Stimuli-responsive polymers can be triggered by various stimuli (*e.g.*, temperature, pH, ionic strength, redox reactions, light, shear stress, enzymes, *etc.*) and change their physical properties, which can lead to dramatic changes in the macroscopic behaviour of polymer solutions, semisolid and solid formulations thereof.[1] This permits also a certain control over properties of particles, implants and other formed bodies made of such polymers by environmental conditions that are characterized by changes in wetting properties of surfaces, charge density, topography, porosity, swelling and others, which raised a strong interest in biomedical fields, such as drug delivery and tissue engineering.[2] Particularly biomedical application of stimuli-responsive polymer systems permits mostly a very small range of stimuli like pH, ionic strength and others, which restricts application of such systems due to the fact that proteins may denature and cells may become damaged if the stimuli are beyond physiological limits. On the other hand, protein and cells can withstand moderate changes in temperature between freezing of water and 42 °C for limited period of time without damage.[3] Interestingly, several types of polymers express thermoresponsive properties in this temperature range, like poly-*N*-isopropylacrylamide (PNIPAM) that has been exploited to prepare coatings on cell culture dishes to obtain cultured cells and cell layers by decreasing the temperature, only.[4] Traditionally, cells need to be harvested from culture dishes by the use of enzymes like trypsin. Proteases enzymatically degrade cell adhesion receptors and adsorbed and secreted extracellular matrix (ECM) proteins, which leads to cell separation.[5,6] However, this nonspecific proteolysis impairs cell function, since different cell surface receptors, transport proteins and ECM are damaged during this process.[7] In addition, integrity of confluent cell layers, applicable for therapeutic and other purposes, is getting lost and must be re-established then later on. Opposed to this harmful enzymatic treatment, cell sheets recovered from thermoresponsive polymer surfaces will retain their structure and function. Many membrane proteins, most of their ECM and especially the cell-to-cell junctions are preserved.[8] The intact ECM of recovered cell sheets enables the re-attachment to various surfaces, like another culture dish or cell sheet, wound sites and host tissues, without the use of sutures.[5,9,10] Hence, the method allows the control over spatial distribution of cells by layering sheets derived from different cell types or the manufacture of 3D tissue constructs by layering monolayer cell sheets.[11] Thermoresponsive materials can be applied in the field of tissue engineering and regenerative medicine in different areas. Depending on the material, they can be used as a hydrogel, as injection-based *in situ* gelling material, for 3D printing or with regard to cell sheet engineering, as modification of a biomaterial surface, which will be lined out in more detail in further sections of this review. Hence, in this review, we summarize the state-of-the-art in the area of thermoresponsive polymers and their biomedical applications,

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especially in the area of tissue engineering for the fabrication of cell sheets, but also hydrogels for 2D or 3D tissue replacement or repair.

2. Mechanism of protein adsorption and cell adhesion

In the field of biomedical application of biomaterials like implants and tissue engineering, protein adsorption and cell adhesion are important processes that determine the success of neoformation and regeneration of tissues on biomaterial surfaces.[12] Protein adsorption and cell adhesion are complex phenomena and heavily depending on surface properties of biomaterials, cell type, and environmental conditions (*e.g.* tissue culture fluids, serum, plasma, blood, *etc.*) (see for example Altankov *et al.*, 1996[13] and Bauer *et al.*, 2013[14]). Protein adsorption is preceding cellular adhesion either in blood or tissues after exposure of any biomedical device or implant, which can be followed by diverse activation-dependent processes like coagulation, complement activation, inflammation, but also, cell adhesion.[15] Cell adhesion is typically followed by cell activation related to morphological changes, proliferation and differentiation of tissue cells.[16,17] That makes protein adsorption essential for cell and tissue colonization on material surfaces. Understanding the interaction between proteins and the surface they adsorb onto is crucial to manufacture surfaces with desired effects on cells and tissues for therapeutic and biotechnological applications.

Proteins—also known as polypeptide chains—are macromolecules composed of amino acids with side groups that may have either positive or negative charges or that are polar or apolar.[18] That makes proteins both amphiphilic and amphoteric. The latter fact is related to the observation that most proteins have a net negative or positive charge at physiological pH value, while the amphiphilic character is related to the polarity of proteins. Both makes them prone to adsorb at interfaces.[18] Hence, proteins can undergo a wide range of physicochemical interactions with other molecules and surfaces that are driven by interfacial energy differences, increasing entropy, Coulomb and other interactions, which are summarized in Fig. 1.[18–20]

It is important to note that protein adsorption is often driven by interfacial energy differences and it can only occur, if the Gibbs free energy (ΔG), defined as $\Delta G = \Delta H - T\Delta S$, decreases ($H$: enthalpy; T: temperature; S: entropy).[18] These differences are found, when hydrophobic polymers or other surfaces contact aqueous solutions, which drives protein adsorption to increase entropy of the water phase. Water molecules shielding the hydrophobic surface and additionally water molecules from hydrophobic amino acid residues (*e.g.* lysins, tryptophan, *etc.*) of the protein molecules are released. In summary, these processes result in a decrease of the Gibbs free energy of the system and hence lead to spontaneous protein adsorption.[21] Furthermore, electrostatic interaction can promote protein adsorption when the sign of net charges is opposite on surface and proteins, which fits to the observation that positively charged materials adsorbed large amount of proteins.[22] In contrast, if the net charge is equivalent, protein adsorption is hindered. Hydrophilic surfaces that form systems of low interfacial energy with the surrounding aqueous phase, promote the adsorption of a thin water layer on the substratum, which generates a very strong repulsive barrier known as hydration force[23] and therefore hinder protein adsorption. A further repulsive force that can play a role in control of protein adsorption is found, when surfaces are covered by hydrophilic macromolecules of certain chain length. Adsorption of other molecules on such a surface is required to change chain conformation of macromolecules from a state of high degree of freedom of mobility to that of a reduced, compressed, which causes a decrease in entropy and thus also a raising of the Gibbs free energy of systems, which is unfavorable.[24] Hence, such kind of layers form a repulsive barrier for protein adsorption, which is depended on the size and density of macromolecules on the surface and the size of proteins.[25,26] In general, protein adsorption is an important prerequisite for cell adhesion, when specific proteins that interact specifically with cell adhesion receptors facilitate the adhesion process (*e.g.*, the presence of fibronectin on surfaces enhances cell adhesion and spreading drastically).[27]

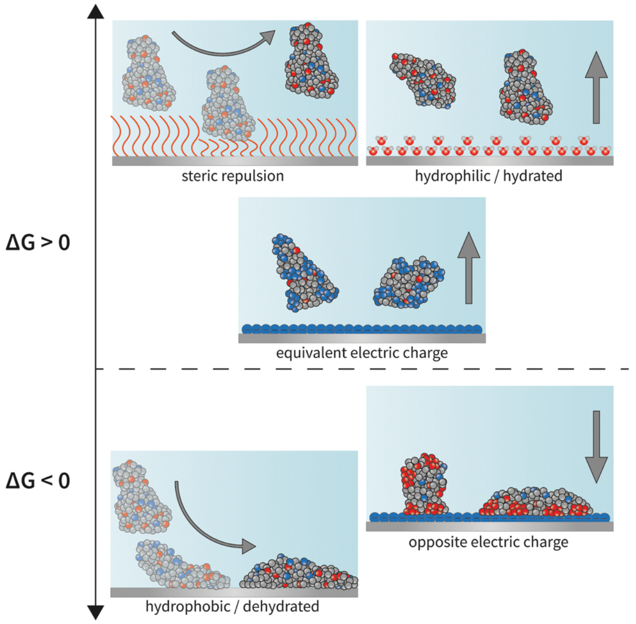

Fig. 1 Surface/protein properties and their resulting Gibbs free energy, hindering or facilitating protein adsorption, respectively. For $\Delta G < 0$, protein adsorption is promoted, for $\Delta G > 0$, protein adsorption is hindered. Surface wettability (hydration forces), electrostatic interaction (Coulomb forces) and steric repulsion of polymer chains are important properties that influence protein adsorption to a surface.

Cell adhesion on solid surfaces is a complex phenomenon; therefore, this review will only briefly explain the underlying mechanisms behind it. For more mechanistic consideration about basics in cell adhesion, the reader is referred to the work of other authors (*e.g.* Bongrand *et al.*, 1982).[28] Cell adhesion is imperative for tissue cells to survive since they are adhesion-dependent and undergo otherwise controlled cell death, which is called apoptosis. This is a regulatory mechanism during embryogenesis, but also in adult organism to control cell survival during reorganization of tissues like mammary gland after stopping lactation, removal of infected cells, *etc.*, but also cells detached by traumata, *etc.*[16] Adhesion is also required for processes like proliferation and differentiation of cells.[29] This is achieved by occupation of cell adhesion receptors like integrins by protein ligands from extracellular matrix proteins, but also proteoglycans followed by signal transduction *via* mitogen-activated protein kinases and other intracellular signaling pathways.[30–32] These proteins like collagens, glycoproteins such as fibronectin, laminin, vitronectin and others are either actively secreted by the cells or delivered from serum that contains for example fibronectin and vitronectin.[33] Physicochemical properties of substrata, such as surface charge density, wetting properties, modification with specific chemical functionalities and state of hydration control the adsorption process of these proteins and hence subsequent cellular attachment, growth and differentiation.[34] Notably, highly hydrophobic and hydrophilic surfaces, respectively, do not support cell adhesion due to conformational changes of proteins by hydrophobic interaction or suppression of protein adsorption on hydrophilic surfaces, which is followed by lack or reduction of cell adhesion and subsequent reactions.[35] In addition, highly hydrophilic substrata suppress protein adsorption and cell adhesion due to repulsive hydration forces.[36] Additionally, modification of substrata with mobile, hydrophilic molecules like PEO leads to reduction or suppression of protein adsorption and cell adhesion, which provides also further control on cell adhesion processes.[37] *In vitro* culture of adhesion-dependent, mammalian cells for a variety of purposes, including tissue engineering, leads to strong cell attachment and formation of confluent monolayers of cells on substrata like plasma-treated tissue culture polystyrene. Cells must either be mechanically scrapped-off or detached by application of enzymes, which both damages cells. Hence, it would be desirable to avoid such harsh procedures and use changes in physicochemical properties of culture substrata to promote or abolish protein adsorption and thus also releasing single cells or cell monolayer ''sheets'' from the culture substratum by changing environmental conditions like temperature. Hereafter, thermoresponsive polymers immobilized on culture vessels or other biomedical devices (*e.g.* implant materials) that change their physical properties from a dehydrated adsorptive state to a hydrated, repulsive state may be a useful tool to achieve this purpose, which will be addressed in the following chapters.

3. Thermoresponsive polymers

Polymers that change their solubility in dependence of the environmental temperature, are called thermoresponsive. The solubility change is accompanied by a conformational alteration in the polymers structure. The temperature, at which the polymeric structure or the solubility changes, is called transition temperature. Since the solubility changes at this point, it is also called critical solution temperature. Thermoresponsive polymers are divided into two groups: either polymers with an upper critical solution temperature (UCST) or polymers with a lower critical solution temperature (LCST). Polymers possessing an UCST behaviour are insoluble in water below this temperature and become soluble above it. UCST behaviour is enthalpy-driven. In aqueous solutions, it requires strong supramolecular interactions, which are weakened upon heating, which leads to phase separation.[38] There are fewer publications regarding UCST in aqueous solutions, since it is more challenging to achieve this behavior[39] at physiological conditions. The main types of reported polymers with UCST behaviour in water are zwitterionic polymers.[40,41] Seuring and Agarwal (2012) published a comprehensive review on polymers with UCST behaviour in aqueous solutions and explain comprehensively the basics, applications and limitations of several polymers.[39] Hence, in this review the focus is on polymers possessing a LCST, which presents the exact opposite effect to UCST.

Polymers with a LCST are completely miscible in a solvent below the transition temperature and phase separation occurs above it. They change their conformation from a rather random coil form to a collapsed, more globular form.[42] The solubility change is due to the fact, that it is energetically more favourable. Below the LCST, solubility occurs because of extensive hydrogen bonding interactions with the surrounding water molecules and restricted intra- and intermolecular hydrogen bonding between polymer molecules.[43,44] Based on the hydrophobic effect and the Gibbs equation $\Delta G = \Delta H - T\Delta S$ (G: free energy, H: enthalpy, S: entropy), phase separation is more favourable when increasing the temperature. The entropy increase of water as a solute is the main driving force, because water is less ordered when the polymer is not in solution, which results in a higher entropy term.[45] The transition of a polymer with LCST is observable, since a polymeric solution below the LCST is transparent and homogenous, but above the LCST it becomes cloudy. Therefore, the LCST is also referred to as cloud point. The LCST of thermoresponsive polymers is affected by the nature of the substitute groups, chain length and molecular weight. In addition, LCST is also influenced by three additives such as salt concentration, co-solvents and surfactants due to the additives, which will affect the hydrogen bonding interactions between polymers and solvent.[46] In this review, we focus on several important thermoresponsive materials, such as, poly(*N*-isopropylacrylamide), derivatives and copolymers of poly(*N*-isopropylacrylamide), Pluronics, ELP V5-120 and poly(*N*-vinylcaprolactam) (Fig. 2).

3.1. Poly(*N*-isopropylacrylamide), its co-polymers and derivatives

The most extensively studied thermoresponsive polymer is poly(*N*-isopropylacrylamide) (PNIPAm) (Fig. 2a). There are several publications available, describing PNIPAm comprehensively. In 1967, the thermal phase transition behaviour of PNIPAm was firstly reported by Heskins and Guillet.[47] In 1992, H. G. Schild presented a comprehensive review of PNIPAm, regarding chemistry, theory and applications.[48] The popularity of PNIPAm is

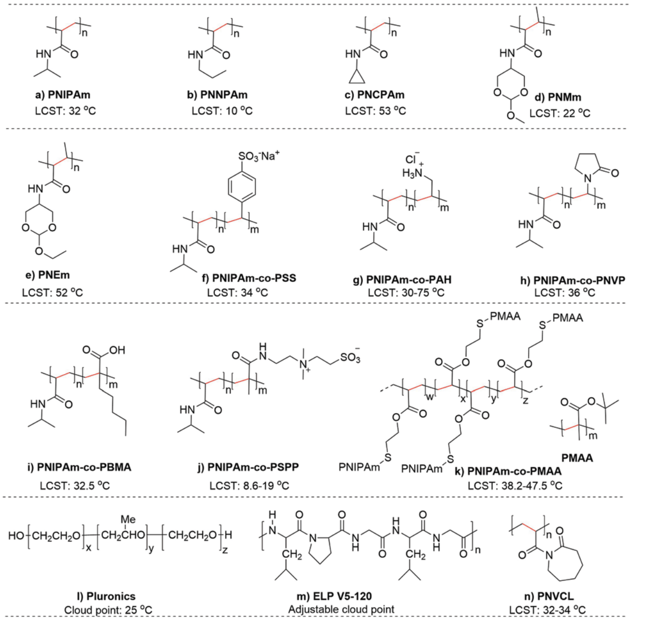

Fig. 2 Chemical structure of (a) poly(*N*-isopropylacrylamide); (b–k) Derivatives of poly(*N*-isopropylacrylamide); (l) pluronics, showing ethylene oxide (EO) and propylene oxide (PO), whereas *x*, *y* and *z* represent the variable amount of EO(*x*, *z*) and PO(*y*); (m) ELP V5-120; (n) poly-(*N*-vinylacprolactam).

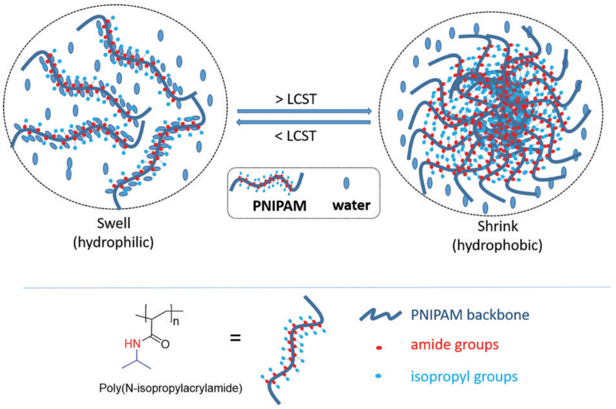

Fig. 3 Graphical representation of thermoresponsive LCST behaviour of PNIPAm.

due to its sharp phase transition in aqueous solution at 32 °C, its LCST is independent of molecular weight and concentration as well as its excellent biocompatibility.[49–52] The LCST at a point near physiological conditions makes it favourable for biomedical applications. Therefore, it attracted increasing interest upon tissue engineering and drug delivery fields and encouraged several publications, especially as reviews in the field of thermo-responsive polymers.[1,52–56]

When passing the LCST, besides conformational (coil-to-globule)[42,57] and solubility changes, PNIPAm alters its wettability. PNIPAm is an amphiphilic polymer, possessing both, hydrophilic (amide groups) and hydrophobic (isopropyl groups) chains. The conformational change to a globule form buries most of the amide groups, which releases a significant amount of water, and hides the hydrophilic groups and exposes the hydrophobic ones, respectively (Fig. 3).[42] This process is reversible and by lowering the environmental temperature below the LCST, the PNIPAm chains extend to their coil form, rehydrate and regain solubility and wettability. Since there are several publications stating that PNIPAm is hydrophobic above the LCST, Pelton *et al.* (2010) published a small communication declaring and explaining that the polymer is never hydrophobic.[58] Indeed, this mechanism is used for the cultivation and harvest of cells onto PNIPAm modified surfaces.

By virtue of its various advantages, developing efficient, controllable and green protocols for the synthesis of PNIPAm are needed. Several methods are available to characterize synthesized PNIPAm. The chemical structure can be determined by NMR[59] and FTIR.[60] In addition, the molecular weight can be measured by gel permeation chromatography (GPC).[61] Dynamic light scattering can be used to determine the LCST.[60]

During the last decades, there were established generally five methods for synthesizing PNIPAm using diverse mechanisms (Table 1). For the first method,[62] the reversible addition-fragmentation chain transfer (RAFT) polymerization offers a number of practical advantages, as it is remarkably tolerant toward a wide range of functional groups, including hydroxyl, carboxyl, and ionic groups and it can be carried out in organic solvents as well as in water. It is categorized as a ''living'' polymerization, as it relies on the equilibrium of dormant and active chains. For the RAFT polymerization, a chain transfer agent (CTA) and an initiator agent are needed. Initiator agents such as (*E*)-2,2′-(diazene-1,2-diyl)bis(2-methylpropanenitrile) (AIBN) play an important role, because they initiate the reaction by generating radicals. In the presence of radicals, CTA (*e.g.* thiocarbonylthio compounds) induce reversible addition fragmentation transfer reactions to create an equilibrium between ''active'' propagating radicals and ''dormant'' CTA-terminated chains that can become active again. This means that RAFT allows to control the polymerization degree by varying the ratio of initiator agent, CTA and monomers.

Atom transfer radical polymerization (ATRP) is a polymerization technique that offers good control over polymer molecular weight and polymer design.[63–65] This method can be carried out in both organic solvents and aqueous media *via* metal-catalysis. Polymerization of the monomer is achieved by the controlled activation of monomer/initiator molecules by the metal catalyst. The metal catalyst is oxidized or reduced and therefore generates or absorbs a radical by complexation with the initiator. This leads to active/dormant chains. Once a chain was activated, it starts propagating with available monomers to polymer chains.[63] The equilibrium between propagating and terminating chains is quite important, because it determines chain length and molecular weight of the resulting polymer. The regulation of active/inactive chains allows control over the polymerization.[66] It is a versatile method that can be performed under mild conditions. However, ATRP generate radicals by an inner-sphere process that requires a high activation. ATRP of acrylamides can be problematic because of complexation of the amide group to the copper catalyst, which can lead to deactivation of the catalyst.

Table 1 Overview of most common polymerization methods of *N*-isopropylacrylamide (NIPAm)

Methods	Conditions	Characteristics	Ref.
RAFT	Initiator, chain transfer agent (CTA)	Controllable molecular weights; tolerant groups; react at various solvent; easy purification; need to remove CTA	62
ATRP	Catalyst copper metal	Inner-sphere radicals process	63–66
SET-LRP	Catalyst copper metal; N-ligand	Outer-sphere SET; narrow molecular weight distribution; efficient reaction; tolerant different solvent	67
Redox initiation	Initiator, accelerator	Depends on buffer, unstable molecular weight	68–70
Ionic polymerizations	Metal alkyl/transition metal halide catalysts	Amorphous PNIPAm; insoluble in aqueous solution as well as polar organic solvent	48

In 2006, single-electron transfer-living radical polymerization (SET-LRP) in the polar media was established by Percec *et al.* (Table 1).[67] The outer-sphere single-electron transfer process involved in this new polymerization has a very low activation energy. Due to this, the reaction can be controllable performed at room temperature or below, with a very small amount of copper as metal catalyst in polar solvents (*e.g.* water, alcohols), dipolar aprotic solvents and ionic liquids. This process provides an ultrafast synthesis of ultrahigh molecular weight polymers from functional monomers.

Another method is redox polymerization of NIPAm using ammonium persulfate or potassium persulfate as the initiator and sodium metabisulfate or *N*,*N*,*N*′,*N*′-retramethylethylenediamine (TEMED) as the accelerator.[68–70] This method suffers from some drawbacks since it depends on buffer to ensure a constant pH, otherwise much greater polymerization degree is obtained. In 1959, Schild *et al.* discovered a novel method of ionic polymerization to produce crystalline PNIPAm *via* "metal alkyl/transition metal halide catalysts".[48] This material is insoluble in aqueous solution as well as in all other typical polar solvents for amorphous PNIPAm, that means this method might be unsuitable to be applied in the biomedical field.

Beside PNIPAm, derivatives of PNIPAm are also very important, especially because of the possibility to adjust the LCST of the thermoresponsive materials *via* polymerization with diverse monomers (Fig. 2b–e) or copolymerizing with different blocks (Fig. 2f–k), *e.g.*, hydrophilic and hydrophobic groups. The synthesis methods for derivatives of PNIPAm were reviewed recently by the groups of Schild *et al.*, Roy *et al.* and Rzaev *et al.*[48,53,71] Therefore, we mainly summarize here their phase transition behaviour properties. The phase transition behaviours of poly(*N*-*n*-propylacrylamide) (PNNPAm) (Fig. 2b)[228] and poly(*N*-cyclopropylacrylamide) (PNCPAm) (Fig. 2c)[229] are significantly different from PNIPAm.[72,73] The different hydrophobic monomers affect the LCST. A similar phenomenon is also observed between poly(*N*-(2-methoxy-1,3-dioxan-5-yl) methacrylamide) (PNMm) (Fig. 2d) and poly(*N*-(2-ethoxy-1,3-dioxan-5-yl) methacrylamide) (PNEm) (Fig. 2e).[74] In order to obtain novel multifunctional materials, there is a need to synthesize specific copolymers.[48,53,71] In 1999, Okano *et al.* established thermoresponsive drug delivery from polymeric micelles *via* copolymerization of PNIPAm and poly(butylmethacrylate) (PNIPAm-*co*-PBMA, Fig. 2i).[75] In 2002, Arotçaréna *et al.* reported on a double thermoresponsive material obtained by copolymerization of the nonionic monomer *N*-isopropylacrylamide (NIPAm) and the zwitterionic monomer 3-[*N*-(3-methacrylamidopropyl)-*N*,*N*-dimethyl] ammoniopropane sulfonate (SPP) *via* RAFT polymerization, where PNIPAM exhibit LCST and PSPP exhibit UCST in water, respectively (Fig. 2j).[41] The copolymer remained in solution in the full temperature range from 0 to 100 °C. Moreover, random copolymers have been synthesized *via* copolymerization of NIPAm with hydrophilic monomers, including sulfate groups and ammonium groups, *via* free radical polymerization reaction (Fig. 2g and 4f).[76] In 2013, Luo *et al.* reported a thermo- and pH-responsive brush-shaped grafted copolymer (Fig. 2k).[77] The resultant nanoscale copolymer micelles exhibited pH-triggered thermoresponsive behaviour, with low critical solution temperature (LCST) about 38.2–47.5 °C. In 2020, Fundueanu *et al.* synthesized a thermoresponsive material possessing a sharp phase transition at 36 °C *via* free radical polymerization of poly(*N*-isopropylacrylamide-*co*-*N*-vinylpyrrolidone) (poly(NIPAm-*co*-NVP)) with a co-monomer molar ratio in copolymer of 91.5/8.5 (NIPAAm/NVP) (Fig. 2h).[78] The adjustable LCST behaviour of the derivatives of PNIPAM and copolymers indicated that it is highly promising to be applied in biomedical field, because the transition temperature can be tuned. This might be relevant for different tissue environments.

3.2. Other thermoresponsive polymers

In addition to PNIPAm and its co-polymers or derivatives, Poloxamers, elastin-like polypeptides (ELP) and poly(*N*-vinylcaprolactam) (PNVCL) are popular thermoresponsive polymers that are used in the biomedical field.

Poloxamers are co-polymers of poly(ethylene oxide) (PEO) and poly(propylene-oxide) (PPO). They also show LCST behaviour, whereas the LCST can be adjusted by the composition of the co-polymer in between the range from 10–100 °C.[79] PEO is highly soluble in water up to temperatures of 85 °C, while PPO is hydrophobic.[80] Preparing co-polymers with different ratios of PEO to PPO, the transition temperature and solubility can be adjusted. At the transition temperature, solutions containing a critical amount of Poloxamers undergo a dramatic change in viscosity. This behaviour is also described as reverse thermal gelation (RTG). Below their LCST, solutions containing PEO–PPO co-polymers have a low viscosity (*e.g.*, which is favourable for injections). With increasing temperature and above their LCST, the viscosity increases drastically. Ideally, they form a semi-solid gel at body temperature.[81] The composition of PEO and PPO to tri-block polymers (PEO–PPO–PEO) (Fig. 2l) with different hydrophilic/hydrophobic segments that show reverse thermoresponsive properties,[81] facilitates their application in

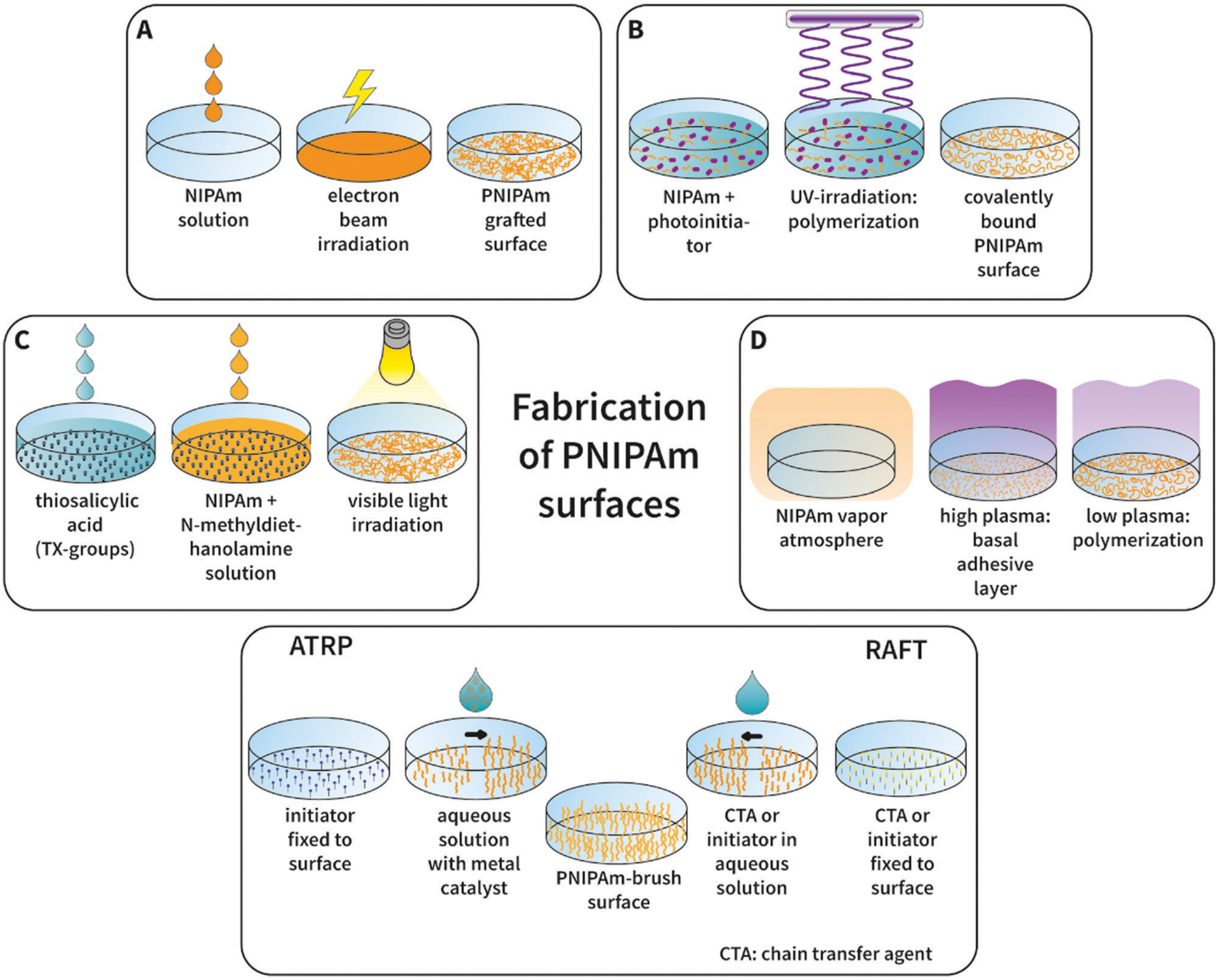
A
NIPAm solution
electron beam irradiation
PNIPAm grafted surface
B
NIPAm + photoinitia-tor
UV-irradiation: polymerization
covalently bound PNIPAm surface
C
thiosalicylic acid (TX-groups)
NIPAm + N-methyldiet-hanolamine solution
visible light irradiation
Fabrication of PNIPAm surfaces
D
NIPAm vapor atmosphere
high plasma: basal adhesive layer
low plasma: polymerization
ATRP
RAFT
initiator fixed to surface
aqueous solution with metal catalyst
PNIPAm-brush surface
CTA or initiator in aqueous solution
CTA or initiator fixed to surface
CTA: chain transfer agent

Fig. 4 Fabrication of PNIPAm-modified cell culture Petri dish surfaces. (A) Electron beam irradiation as published by Okano *et al.* Petri dishes are commercially available. (B) NIPAm is prepared with photo initiator and grafted to the surface and polymerized *via* UV irradiation. Surfaces with covalently bound PNIPAm are achieved. (C) Petri dishes immersed in thiosalicylcic acid to generate TX-groups on surface. In the next step, solution of NIPAm and *N*-methyl diethanolamine are filled into Petri dishes. Visible light is used for polymerization with TX-groups as anchoring points for PNIPAm chains on surface. (D) NIPAm vapor atmosphere in low vacuum is prepared. A high energy plasma is applied to create a thin basal anchoring layer. Polymerization and chain growth occur in low energy plasma. ATRP and RAFT: polymerization methods for the creation of PNIPAm brush surfaces.

controlled drug release,[82] tissue engineering[83] and wound dressing fields.[84] The commercially available and most widely used co-polymer of PEO–PPO is the triblock (PEO–PPO–PEO), also known as Pluronics®. A minimum concentration in solution of 15–20% is necessary to achieve a solution (sol)–gelation (gel) transition and further adjustment of the concentration allows to create materials with required viscosities. The LCST for the well-known Pluronic F127 is at around 30 °C, and hence in the physiological range. There are several investigations concerning the driving forces of RTG behaviour. Intrinsic changes in micellar properties, the formation of three-dimensional networks and, similar to PNIPAm, the gain in entropy are proposed as reasons for the gelation.[85–88] In 2013, Basak *et al.* discovered that the reverse thermoresponsive behaviour of PEO–PPO–PEO, driven by the entropy gain provided by the release of bound water molecules structured around the hydrophobic segment, leads to their ability to self-assemble into diverse liquid crystalline topologies.[89,90]

Among all the PEO–PPO–PEO types, Pluronic F127 (PF127), $(EO)_{99}$–$(PO)_{65}$–$(EO)_{99}$ is one of the commercially available high molecular weight block polymers, which are made by the sequential addition of propylene and ethylene oxides *via* propylene glycol initiator, at conditions of elevated temperature and pressure and in the presence of a trace of a basic catalyst such as sodium or potassium hydroxide (Fig. 7).[91] PF127, molecular weight of 12500, is a white solid with a melting point of 56 °C and solubility in water. PF127 is more soluble in cold water, since higher temperature could disrupt the hydrogen bonding (the hydrogen bonding between O from PF127 and H from water). When the concentration of PF127 is above 20% in water at 25 °C, it will form a gel. PF127 attracted much interest due to its reversible sol–gel transition behaviour in aqueous solution. In addition to thermoresponsive properties, PF127 has several advantages, such as excellent biocompatibility, enhancement of protein stability, lack of inherent myotoxicity and immunotoxicity.[92] Because of these special properties, PF127 has been widely used in topical, ocular, nasal and rectal drug delivery.[93]

The third type of popular thermoresponsive polymers are the elastin-like polypeptides (ELP, Fig. 2m). Elastin is a structural extracellular matrix protein that is present in all vertebrate connective tissue, such as arteries, skin, lung, and ligament.[94,95] Tropoelastin, the soluble precursor of elastin, is composed of alternating hydrophobic and hydrophilic crosslinking domains. Once tropoelastin is secreted into the extracellular space, insoluble elastin is created by strong crosslinking through the action of lysyl oxidase.[96] ELPs are repetitive artificial polypeptides derived from recurring amino acid sequences –Val-Pro-Gly-Xaa-Gly– found in the hydrophobic domain of tropoelastin (Val: valine; Pro: proline; Gly: glycine; Xaa: any amino acid other than Pro). The reversible thermoresponsive polypeptides (Fig. 9) are attractive for the use in tissue engineering and drug delivery fields for several reasons.[97] Firstly, ELPs can be genetically encoded. That means, a controlled synthesis, precisely to specific molecular weight and amino acid sequences on demand, is possible, even in a heterologous host (*e.g.* bacteria or eukaryotic cell). Secondly, ELP can be easily expressed at high yield (100–200 mg L^{-1}) from Escherichia coli and rapidly purified by exploiting their phase transition behavior.[98,99] Thirdly, they are biocompatible, biodegradable and non-immunogenic.[100] Due to their important functional role as component of the native extracellular matrix, ELPs have attracted increasing interest in drug delivery and tissue engineering.[101,102]

Another thermoresponsive polymer that has gained attention among researchers over the past years, is poly(*N*-vinylcaprolactam) abbreviated as PNVCL (Fig. 2n). It shows similar characteristics like PNIPAm, such as a similar LCST behaviour between 32 and 34 °C[103,104] and a reversible swelling to collapsing transition (similar to coil–globule transition in PNIPAm) at the LCST in water.[105,106] PNVCL is only second in popularity among thermoresponsive polymers, which is most likely due to the difficulties to polymerize NVCL in a controlled manner. The first report of synthesizing PNVCL was published by Solomon *et al.* in 1968[107] in English language. Since then, several researchers focused on the synthesis of PNVCL.[108–112] with defined molecular weight and dispersity, because they influence the thermoresponsive properties of the polymer. Mainly, the above-mentioned RAFT method is used for controlled polymerization of NVCL.[113] Many studies also focused on the biocompatibility of PNVCL. Vihola *et al.* (2005) described in a comprehensive publication the biocompatibility of PNVCL.[114] They show that PNVCL is generally biocompatible. However, cytotoxicity is slightly enhanced above the LCST at 37 °C. For a comprehensive overview and detailed information on PNVCL, the review written by Cortez-Lemus and Licea-Claverie (2016) is recommended.[115] Additionally, Rao *et al.* (2016) described in their publication the biomedical application of stimuli-responsive PNVCL gels.[113]

Not all of the aforementioned polymers can be successfully applied in all of the kind of biomedical applications. We will describe in the following sections of this review in which way these polymers are applied in the field of tissue engineering and other biomedical applications.

4. Fabrication methods of PNIPAm-grafted thermoresponsive surfaces

The most established technique to prepare thermoresponsive surfaces consisting of PNIPAm is based on electron beam (EB)-induced polymerization. It was introduced by Yamada *et al.* (1990).[116] They prepared a solution of the monomer NIPAm, dissolved in isopropyl alcohol, which was added to tissue culture polystyrene (TCPS) wells. Polymerization and grafting of NIPAm on the surface are achieved by electron beam irradiation. After thorough cleaning, this process results in the formation of a thin polymer layer on TCPS (Fig. 4A). The surfaces exhibit a thermoresponsive behaviour and are successfully used to harvest cells through change of temperature. Up to day, this technique has been revisited several times and further instructions and characteristics of the resulting surfaces have been published.[117–120] Covalently bound to TCPS, the optimal thickness of PNIPAm layer for thermoresponsive cell adhesion and detachment is in the range of 15–20 nm.[120] By contrast, PNIPAm covalently bound to glass surfaces has an optimal thickness of only 3.5 nm for the thermoresponsive behaviour towards cells.[121] Furthermore, a more rapid cell detachment is achieved on porous thermoresponsive surfaces, because the hydration of PNIPAm chains is facilitated.[122] These facts listed above show that many factors influence the functionality of PNIPAm-grafted surfaces with regard to temperature-controlled adhesion and detachment of cells. As of today, this technique is applied for the mass fabrication of thermoresponsive cell culture dishes (TRCD), which are commercially available and used in cell sheet engineering. The applications in regenerative medicine and cell sheet engineering of these grafted surfaces are discussed later in Section 5 of this review.

Nevertheless, there is one major drawback of EB irradiation, because the equipment required is expensive and complicated and common laboratories are rarely equipped with such machinery. Hence, several other preparation methods have been developed for making of PNIPAm-grafted, thermoresponsive surfaces, such as plasma irradiation, UV irradiation and visible light irradiation along with a photo initiator (Fig. 4). The plasma irradiation (Fig. 4D) enables the fabrication of surfaces that exhibit almost no thickness-dependent cell-repellent effect.[123] For the surface modification, a NIPAm-monomer vapor atmosphere in a low vacuum is formed. High plasma power is utilized to fabricate a basal adhesion-promoting layer. Onto this layer, functional polymer deposition is carried out at reduced plasma power.[124] Several studies have prepared such thermoresponsive surfaces, fabricated by plasma activation.[125–127] This method produced surfaces that achieved the desired results in cell culture and detachment experiments, comparable to surfaces fabricated by EB irradiation.

Furthermore, ECM proteins successfully detached together with the cell sheet.[123] The second alternative to EB irradiation is grafting PNIPAm onto surfaces *via* UV irradiation (Fig. 4B). Morra *et al.* (1996)[128] firstly grafted PNIPAm onto polystyrene dishes by the use of UV light. They combined the monomer NIPAm with a photo initiator, benzophenone, dissolved in 2-propanol solution. By exposure to UV irradiation, the monomer is polymerized and covalently grafted to the PS dish surface. They achieved a thermoresponsive effect at around 10 °C and harvested cells successfully in form of sheets. UV irradiation was further used to make patterned surfaces, as demonstrated by Ito *et al.* (1997). In contrast to the work of Morra *et al.*, they utilized a PNIPAm-copolymer with acrylic acid and azido phenyl groups as photo crosslinking unit.[129] In more recent years, Nash *et al.* (2012) used the technique of spin coating to create a thin, UV cross linkable surface of NIPAm copolymerized with a photoinitiator.[130] After a thin film of polymeric solution was coated onto Thermanox™ tissue culture discs, they were crosslinked by UV irradiation. Thirdly, a new method using visible light irradiation (Fig. 4C) was introduced by Fukumori *et al.* (2016). They developed a two-step process using polystyrene (PS) dishes as a substrate.[131] During the first step, thiosalicylic acid, dissolved in concentrated sulfuric acid, is incubated in PS dishes. This allows the manufacture of a modified PS dish with thioxanthone (TX) groups. The TX-PS-surface is then further modified during the second step by adding an NIPAm monomer solution containing *N*-methyl diethanolamine onto these surfaces. Thereafter, by irradiation with visible light, made either by LED or a mercury lamp, the monomer is polymerized and grafted onto the surface. The TX-groups on the PS substratum functions as photo initiator and anchoring units and are responsible initiating the polymerization during irradiation.

Besides the above-mentioned methods to prepare thermoresponsive surfaces, PNIPAm brush surfaces have been fabricated, too. They are of interest, because of the better control of wettability and possible end-group functionalization and chemistry of the PNIPAm molecule.[132] They enable additional applications other than cell sheet engineering, described in a comprehensive review by Nagase *et al.* (2018).[133] Two grafting techniques are mostly used to fabricate brush surfaces, namely atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer (RAFT). They allow precise surface fabrication with control over chain length, film thickness and produce higher grafting densities.[132,134] In contrast to the aforementioned use for the polymerization of PNIPAm, surface-initiated ATRP starts with the immobilization of a halogenated initiator on the substrate surface, followed by the polymerization process (Fig. 4, ATRP). This ensures the fabrication of the polymer film directly on the surface. Cooperstein *et al.* (2015) grafted PNIPAm-surfaces using ATRP for the use in cell culture and detachment.[9] In contrary to the advantage to polymerize PNIPAm in a controlled manner directly on the surface, the use of copper as metal catalyst presents a limitation for biomedical applications of the fabricated surfaces due to the toxicity of copper ions. Therefore, several groups tried to modify the ATRP using different catalysts to overcome this limitation. Conzatti *et al.* (2017) provides a comprehensive overview of several ATRP methods.[132] Besides the ATRP, the RAFT technique is often applied to fabricate brush structures on surfaces. The process of a RAFT-polymerization was mentioned already in section 3. For the surface-initiated RAFT, either the initiator or the chain transfer agent (CTA) must be introduced to the surface before polymerization (Fig. 4, RAFT).[132,135] Surface-initiated RAFT is a well-controlled process, which allows the fabrication of surfaces with a narrow chain distribution and low polydispersity, *e.g.* for PNIPAm-grafted surfaces polydispersity indices of 1.3 were achieved.[136] Furthermore, it can be performed in a wide temperature range, between room temperature and 140 °C. One major advantage, in comparison to ATRP, is the abundance of metal-ions. A comprehensive handbook on the RAFT technique was published by Barner-Kowollik *et al.* (2008).[137]

The aforementioned methods are versatile and generate PNIPAm surfaces suitable for cell sheet engineering. However, they show certain difficulties, *e.g.* use of metal-ions, photo initiators and chemical compounds that are cytotoxic and the expansive machinery. Furthermore, it has certain limitations, as it has been shown that cell adhesion on PNIPAm surfaces is inferior to adhesion on cell culture polystyrene.[138] The layer-by-layer (LbL) technique, described first by Decher *et al.* (1992) and since then used as a versatile, low cost and easy to perform method for making multilayer films, presented a promising alternative.[139] It relies on the alternate deposition of anionic and cationic polyelectrolytes on any charged substrate. By immersing the substrate alternatingly in solutions of oppositely charged polyelectrolytes, with a washing step in between, a multilayer film can be fabricated. The stepwise addition of layers allows control over film thickness. This technique has been used for fabrication of several layers and extensively reviewed.[140,141] However, in the field of thermoresponsive polymers, only little research has been done. Serpe *et al.* (2003) was one of the first groups describing the creation of thermoresponsive multilayers.[142] They combined PNIPAm with acrylic acid (AAc) to form microgels and produced polymeric thin films using poly(allylamine hydrochloride) (PAH) as polycation. Glinel *et al.* (2003) used poly(diethylaminoethylmethacrylate)-*block*-PNIPAm and poly(styrene sulfonate)-*block*-PNIPAm to successfully fabricate thermoresponsive multilayers.[143] In 2005, Jaber and Schlenoff presented the manufacture of polyelectrolyte multilayers, combining PNIPAm with PAH and poly(styrene sulfonate) (PSS).[76] They showed thermoreversible behaviour of their multilayers. Reviewing the literature, it is obvious that the general method for manufacturing multilayers *via* LbL is to graft PNIPAm-*co*-polymers to polyelectrolytes, since PNIPAm is uncharged and cannot be used directly. This is more benefit than limitation, because it allows the combination of thermoresponsive properties with other polymers, *e.g.* biopolymers or polysaccharides.[10,144] This enables the fabrication of multilayers with more favourable adhesion properties than pure PNIPAm surfaces using co-polymers, even allowing the incorporation of biomolecules.[141] Since research in the area of thermoresponsive multilayers is sparse, it still holds great potential for future applications, especially by combining PNIPAm with biopolymers.

5. Application of thermoresponsive polymers in regenerative medicine and tissue engineering

5.1. Cell detachment from PNIPAm surfaces

Cultivation of cells is a prerequisite for all type of fundamental biomedical research but also for producing cells, tissues and organoid-like constructs in the area of regenerative medicine and cancer research. Therefore, cells are seeded onto tissue culture surfaces like flasks, multi-well plates, membranes, *etc.* on which they shall adhere, grow and sometimes also differentiate into the desired tissue. Especially for replacement of epithelial and other two-dimensional tissues, cells cultivated as confluent sheets of monolayers can be used directly.[145] However, this requires a removal or harvesting process, contrary to conventionally used enzyme or scraping process, which does not harm the cells and retains cell–cell-connections.[10,33] The use of thermoresponsive surfaces allows the harvest of unharmed and interconnected cell sheets consisting of a cell monolayer.[146] Cells detach because the substrate they grow on changes its conformation and hydration state.[118,147] For cell sheet engineering, surfaces with polymers possessing a LCST behaviour are most commonly used.[148] Cells are seeded and cultivated at temperatures above the LCST (at around 37 °C), at which PNIPAm chains possess a more globule conformation and expose hydrophobic isopropyl groups.[58] Furthermore, the surfaces are dehydrated, which in summary facilitates protein adsorption on the surface.[32,149] Thereafter, cells adhere mediated through integrin receptors, which bind to the adsorbed proteins. Conventionally, cells would only detach by application of enzymes or cell scrappers. But on thermoresponsive surfaces, by changing the environmental temperature to a value below the LCST (*e.g.*, for PNIPAm around 32 °C), thermoresponsive polymers hydrate and undergo a conformational change, exposing hydrophilic groups,[58,149] which shall lead to cell detachment. However, in 1982 Grinnell *et al.* showed that binding between ECM and cells is still active even at 4 °C,[150] which meant that the mechanism of cell detachment must be due to the weakening of ECM-substrata interactions.[151] ECM proteins, like fibronectin or laminin, are less tightly bound to the surface due to hydration forces.[151,152] While this initiates the process, for complete cell detachment, an active, ATP-consuming reorganization of the cytoskeleton and signal transduction is necessary.[118,153] Adhering cells possess actin-based stress fibers, exerting traction and contraction forces.[154] For adherent cells, an equilibrium between pulling forces of the cytoskeleton and tensile stress of the ECM is reached. But, if the temperature is reduced, hydration eliminates tight bonding between ECM and surface. At this point, pulling forces of metabolic active cells exceed tensile stress of ECM, which leads to cell rounding and detachment from the surface.[118,153] For cell sheets, the same mechanism is applied. However, due to maintained cell–cell-junctions, the pulling forces of the cells lead to a rolling and contraction of the cell sheet,[151] which allows recovery of cell sheets attached to their secreted ECM.[155–157] Fig. 5 illustrates the process of cell sheet detachment. The sheets maintain growth and secretion activities, and substrate adhesiveness, nearly comparable to primary cells.[117,158] Cell type, temperature, adsorbed proteins, and surface properties influence the detachment process on thermoresponsive layers.[118,159] However, there is one drawback for the temperature dependent cell recovery. The low temperatures expose cells to a cold stress that restrains their metabolic activity. Consequently, exposure to low temperatures should be kept to a minimum amount of time and the temperatures should not be too low.[50]

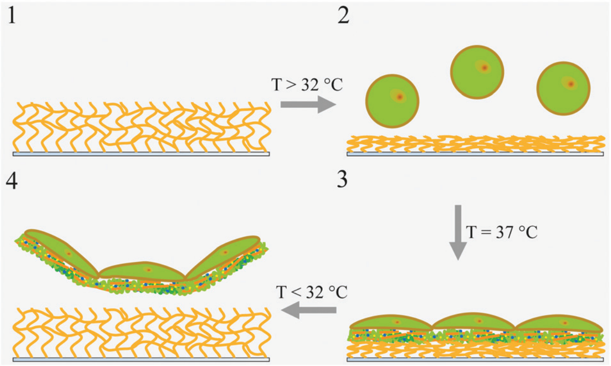

Fig. 5 Scheme for the mechanism of cell detachment on thermoresponsive surfaces: (1) elongated, coil-like surface structure at below 32 °C; (2) temperature raised above 32 °C and seeding of cells onto conformationally changed surfaces, cell adherence facilitated; (3) Cells proliferate and excrete ECM; (4) temperature is reduced to below 32 °C, change in surface properties and detachment of cells, ECM and cell-to-cell-junctions remain intact. Due to contraction forces exerted by actin-based stress fibres, cell sheets roll up.

5.2. Cell sheet tissue engineering using thermoresponsive surfaces

Since Yamada and Okano *et al.* (1990) presented the first cell culture dish modified with PNIPAm on which they successfully detached an intact cell monolayer, several different cell types have been grafted into sheets.[116,160] In recent years, some of these cell sheets achieved clinical approval while others are still under study and clinical approval is still pending. Most thermoresponsive systems, especially surfaces for cell sheet engineering, are based on PNIPAm, as mentioned before.[160–163] Thermoresponsive cell culture dishes (TRCD) are by now commercially available and used as substrate in most of the following publications. Fig. 6 shows an intact cell sheet detached from these commercially available TRCD.

Cell sheets are used in the field of tissue engineering to regenerate, rebuild or replace several kinds of damaged or non-functioning tissues. In recent years, cell sheet engineering was successfully applied to manufacture tissue constructs *in vitro*. Cell sheets can be handled and manipulated while they maintain cell-to-cell junctions and most of their secreted ECM proteins underneath and above the cells. Cell sheets maintain also a certain ‘‘adhesiveness’’, because the ECM is working as a ‘‘glue’’,[149] allowing them to re-attach on surfaces and stacking them to create thick and dense tissues.[160,164] This is due to the presence of ECM proteins, especially glycoproteins like fibronectin (FN).[125] FN has the ability to bind a large number of

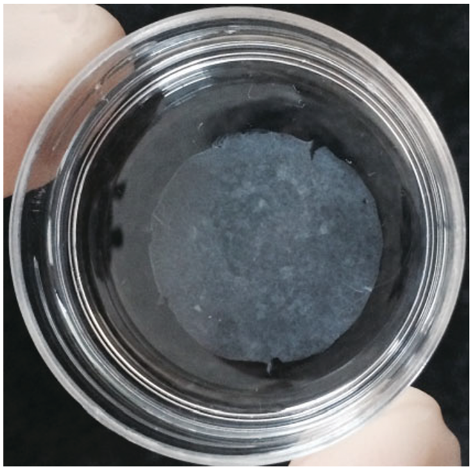

Fig. 6 Intact cell sheet obtained from thermoresponsive PNIPAm-modified cell culture dish. Adopted with permission Ohki *et al.* (2015).[164]

molecules, among them several ECM (*e.g.* proteoglycans, collagen), signaling (*e.g.* growth factors like BMP-2) and cell adhesion molecules (integrins like $\alpha 5\beta 1$).[149,165] This enables cell sheets further to cover wounds without the need of sutures or tissue sealants.[166] The process of cell sheet engineering can be considered as sequence of (Fig. 7[167]): Fabrication of homotypic (A) or heterotypic (B) monolayer (C) or multilayer (D) cell sheets on surfaces grafted with different thermoresponsive polymers Transplantation of single cell sheets (C) to replace epithelia like skin, cornea, *etc.*, use of homotypic- multi-layered cell sheets to replace cardiac muscle tissue (D), and the use of alternating homotypic or heterotypic monolayers to generate more complex liver or other organ tissue substitutes (E).[168] However, most of the tissues replaced contain lower amounts of ECM, *e.g.* epidermis, liver and heart tissue rich in epithelial cells or cardiomyocytes. For ECM-rich tissues, like bone or cartilage, cell sheet engineering cannot provide enough ECM, but may be applicable after longer culture of such combined tissue substitutes.

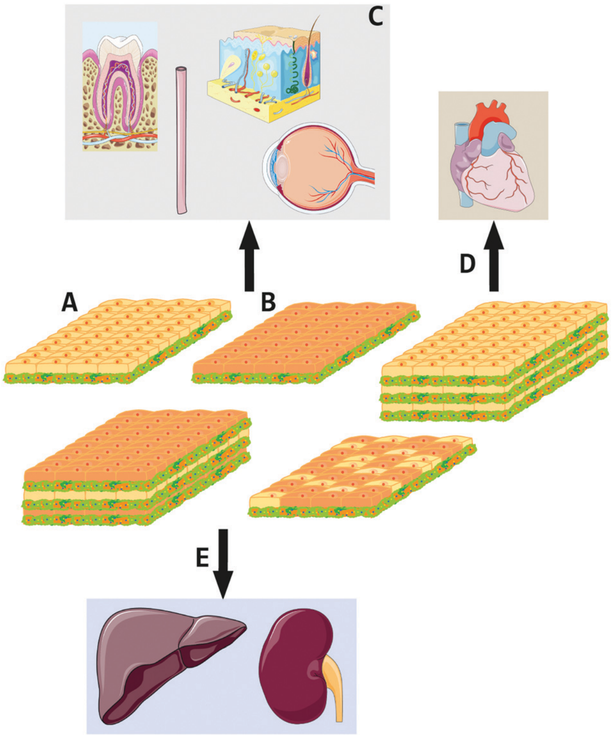

Fig. 7 Principle of cell sheet engineering: (A and B) homotypic cell sheets for the fabrication of tissue substitutes for cornea, oesophagus, skin or periodontal ligaments (C); (D) homotypic multilayer cell sheets as tissue substitute for *e.g.* heart tissue; (E) heterotypic mono- or multilayer cell sheets for the creation of more complex tissues like kidney or liver. Illustration of the organs are kindly provided by Smart – Servier Medical Art underlying a Creative Commons License 3.0.[167]

Corneal epithelial reconstruction is the most prominent example for the use of single cell sheets.[166,169] For patients with damaged cornea, a biopsy of autologous corneal stem cells can be taken. These cells are cultivated into a confluent monolayer of cells, after which the sheet can be harvested and transplanted as cornea replacement into the patients' eye. However, corneal failure caused by a severe trauma or an eye disease can also result in the absence of corneal stem cells. Therefore, autologous epithelial cells are obtained from the oral mucosa epithelium and are transplanted as replacement into the patient's eye. This method results in the successful reconstruction of corneal tissue and restores visual acuity.[170]

Cell sheet engineering (CSE) technology is also available and, in some cases, clinically applied, to patients for oesophagus regeneration after endoscopic submucosal dissection. Patient-derived oral mucosal epithelial cell sheets are cultivated to autologous cell sheets.[171] After temperature-induced detachment of the sheets, they were transplanted onto the ulcer surface of the oesophagus *via* endoscope. The oesophagus surface completely re-epithelialized. The stricture formation normally accompanying a surgical oesophagus treatment was successfully prevented.[172] The use of oral mucosal epithelial cells to repair oesophagus tissue has been studied thoroughly before applying this technique to patients.[173–175] Nasal mucosal epithelial cell sheets were crafted and used for the restoration of the middle ear cavity mucosa. Fig. 8 presents a scheme in which way the procedure of cell harvest, cell sheet fabrication and transplantation into the middle ear cavity is carried out.[176–178] This technique is currently used in medical practice.

Cartilage and periodontal regeneration are achieved by the transplantation of multi-layered cell sheets. Restoration of periodontal tissue could be accomplished by the transplantation of multi-layered cell sheets. Cells, derived from the periodontal ligament, are fabricated into monolayer sheets.[179–181] After temperature responsive cell detachment, obtained cell sheets are stacked to three-layered constructs. The remaining basal layer of ECM on the cell sheets works as an adhesive. These TE constructs have been used in several studies[179,180] and the results indicated the successful regeneration of periodontal tissue and the effectiveness of cell sheet transplantation.[182,183] Therefore, this therapy is currently performed in patients. The regeneration of cartilage tissue, as mentioned above, has been realized in a similar fashion. Chondrocyte sheets are cultivated and detached after successful fabrication of a confluent cell

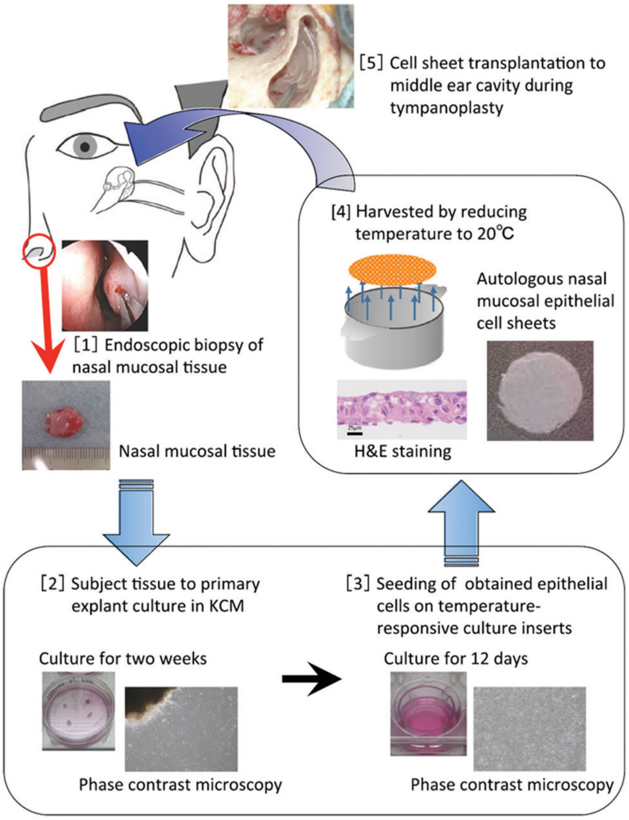
[5] Cell sheet transplantation to middle ear cavity during tympanoplasty
[4] Harvested by reducing temperature to 20°C
Autologous nasal mucosal epithelial cell sheets
H&E staining
[1] Endoscopic biopsy of nasal mucosal tissue
Nasal mucosal tissue
[2] Subject tissue to primary explant culture in KCM
Culture for two weeks
Phase contrast microscopy
[3] Seeding of obtained epithelial cells on temperature-responsive culture inserts
Culture for 12 days
Phase contrast microscopy

Fig. 8 Transplantation of autologous epithelial cell sheets fabricated from nasal mucosal on PNIPAm-modified cell culture dishes into the middle ear cavity for middle ear mucosal regeneration. The scheme shows the step-by-step preparation and transplantation. Adopted from Yamamoto *et al.* (2017).[178] This image is licensed by the aforementioned authors under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

monolayer. Several monolayers are stacked on top of each other to form a multi-layered chondrocyte sheet construct. These constructs are transplanted to the cartilage defect. Several studies on the fabrication and use of chondrocyte sheets for cartilage regeneration have been performed.[184–186] Fig. 9 shows regeneration of a cartilage defect in a minipig study. Three months after the defect was covered with a three-layer chondrocyte cell sheet, it is re-filled with cartilage tissue.[184] Thereafter, repair of human cartilage tissue, with the help of chondrocyte sheets, has been examined.[187] This therapy is clinically applied in patients for cartilage regeneration.

There are several other applications of the cell sheet technology for tissue engineering and regenerative therapies. Dermal fibroblast cell sheets were successfully fabricated to seal air leaks of the lung.[188–190] Furthermore, cell sheets of keratinocytes,[191,192] pancreatic islet cells,[193–195] and mesenchymal stem cells were successfully fabricated and applied.[196,197]

Furthermore, cell sheet engineering is applied to create thick and dense tissues, such as heart or liver tissue. (*e.g.* Fig. 7). As mentioned above, to manufacture thick tissue constructs, cell sheets need to be stacked. They can be stacked homotypic (several sheets of one cell type) or heterotypic (sheets of more than one cell type), depending on the targeted tissue. Hepatocyte and endothelial cell sheets were stacked in alternating fashion on top of each other to create liver tissue. The results showed, that the combination of those two cell sheet types enables the successful creation of hepatocyte tissue with expression of normal hepatocyte functions.[198,199] Stacked cell sheets were transplanted into mice and developed into miniature three-dimensional liver systems.[200] Unfortunately, large scale constructs fail because of insufficient supply of oxygen to cells in the core region of transplant and need further research.[201]

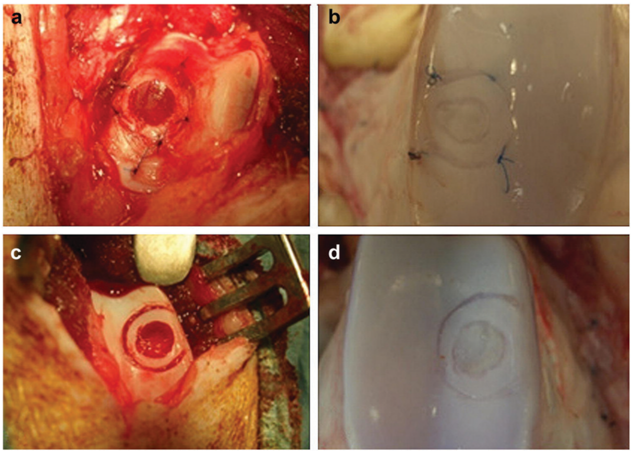

Fig. 9 Transplantation of chondrocyte cell sheets into minipigs. A defect (6 mm diameter, 5 mm deep) was made in the animal's medial femoral condyle, covered with a three-layer chondrocyte cell sheet (a). For the control group (c), defect was not covered with cell sheets. After three months, the defect was filled with cartilage tissue for the cell-sheet group (b), the control group showed insufficient filling of the defect with cartilage tissue (d). Adopted with permission from Ebihara *et al.* (2012).[184]

Based on the cultivation of cardiomyocytes and cell sheets thereof, different cardiac tissues could be fabricated. Pulsatile tubes, cell sheets and multi-layered cell-stacks were successfully cultivated.[202–205] However, in thick heart tissue, consisting of stacks of more than three sheets, cells undergo necrosis because of insufficient oxygen and nutrient supply.[206] This is similar to liver tissue and needs further research to pre-vascularize the cell sheet construct *in vitro.*

The above-mentioned cell sheets were cultivated on solely PNIPAm-grafted cell culture dishes. But, for the fabrication of complex tissues (*e.g.* liver, kidney), several different cell types are needed. For the creation of such heterotypic tissues, patterned, thermoresponsive surfaces were developed. In a three-step process, Tsuda *et al.* (2005) fabricated patterned surfaces with different transition temperatures by co-polymerization of PNIPAm with *n*-butyl methacrylate (BMA) side chains.[207] Firstly, they fabricated a PNIPAm-modified surface in a cell culture dish with the standard electron beam irradiation method. Thereafter they placed onto that PNIPAm-modified surface a metal mask with a hole pattern and filled the dish with a BMA/2-propanol solution. Then, *via* EB irradiation for a second time, BMA was co-polymerized with PNIPAm only in the spots not covered by the metal mask. This allows a temperature-regulated, site-selective cell adhesion. Endothelia cells adhered on PNIPAm-*co*-BMA regions at 27 °C, allowing co-culture with hepatocytes seeded at 37 °C, which adhered on the cell free regions. In this manner, several patterns could be fabricated allowing the creation of heterotypic cell sheets of different cell types for the creation of complex tissues. Ore information about this approach can be found at Nagase *et al.* (2018), who reviewed several methods for the fabrication of such surfaces and their applications.[160]

Another example for the alteration of PNIPAm-modified surfaces *via* co-polymerization is the work of Nitschke *et al.* (2006).[208] They co-polymerized PNIPAm with diethyleneglycol methacrylate (DEGMA). The PNIPAm-*co*-DEGMA surfaces were prepared using a low-pressure plasma treatment. These surfaces showed a transition temperature slightly higher and thus closer to the physiological range. The study shows that human corneal endothelial cells (HCEC) could adhere, spread and proliferate on these surfaces, A harvest of HCEC cell sheets with ECM was achieved by lowering the temperature to 30 °C. Overall, they could show that the co-polymer surfaces were advantageous compared to PNIPAm surfaces due to an efficient and more gentler cell harvesting process.

Recently, Nguyen *et al.* (2019) modified polycaprolactone (PCL) microcarriers with PNIPAm, showing that not only cell culture dishes could be modified with a thermoresponsive surface, but also microcarriers used in cell culture.[209] They immobilized PNIPAm chains onto PCL micro beads *via* amidation reaction. The PNIPAm-conjugated PCL microcarriers showed a non-toxic, biocompatible behaviour and excellent cell attachment of human dermal fibroblasts and mesenchymal stem cells. By reducing the temperature from 37 °C to 30 °C, cells could be detached from the microcarriers. Cells recovered better from the detachment process than after trypsin treatment. They suggested that the system might be predestined for a future use in large scale cell production.

On thermoresponsive surfaces produced *via* layer-by-layer (LbL) technique, Liao *et al.* (2010) cultivated human mesenchymal stem cells (hMSC).[210] They used Poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) modified with PNIPAm chains for the multilayer formation. They divided multilayers by different terminal layers (PAH, PSS or fetal bovine serum). PAH terminated multilayers showed most favourable results for cell adhesion. In general, multilayers enabled thermoresponsive cell detachment. Overall, they stated that these multilayers are very promising for the use in hMSC cultivation. However, publications in this area of research are rare and as presented before, electron-beam-grafted PNIPAm dishes are still the most applied surface for cell sheet engineering.

PNIPAm has not only been used for cell sheet engineering, but also as part of thermoresponsive drug delivery systems. As early as in 1999, Chung *et al.* developed thermoresponsive polymeric micelles constructed using PNIPAm and poly(butyl methacrylate) copolymers (PNIPAm-PBMA).[75] They loaded an anticancer drug inside the PBMA micelle inner core, while PNIPAm chains build the outer shell. Drug release was initiated by heating above the LCST and structural deformation of PNIPAm chains at this point. The loaded micelles showed reversible, thermoresponsive drug release. This research presented promising results for the use of micellar structures made of thermoresponsive polymers as drug delivery systems. In 2013, Luo *et al.* copolymerized PNIPAm with poly(methylacrylic acid), allowing a spontaneous assembly of these copolymers into nanoscale core–shell–corona micelles.[77] They showed stability of the micellar structure under simulated physiological conditions and the thermoresponsive drug release using prednisone as sample drug. Most recently, Fundueanu *et al.* (2019) prepared and researched thermoresponsive microspheres consisting of copolymerized NIPAm with *N*-vynilpyrrolidone (NVP).[78] The Poly (NIPAm-*co*-NVP) possesses a sharp phase transition at body temperature under physiological conditions. They successfully incorporated diclofenac as sample drug and showed for low loaded microspheres and temperature triggered, pulsatile drug release mechanism. These co-polymers based on the thermoresponsive properties of PNIPAm showed promising results for the use as drug delivery systems. For the delivery of hyaluronic acid for osteoarthritis therapy, Maudens *et al.* (2018) modified hyaluronic acid (HA) backbones with PNIPAm side chains.[211] These conjugates are spontaneously forming nanoparticles at body temperature. They showed that one of their HA conjugates is easily injectable, stable, biocompatible and biodegradable, showing a prolonged residence time at the injection site. Tested in an osteoarthritis model in mice, the HA-PNIPAm system exhibited a protecting effect on the epiphysis thickness of the medial tibia. Furthermore, they suggested that the system can potentially be used as delivery systems for peptides, proteins or small molecules. The *in situ* formation of HA nanoparticles introduces a new option for the lubrication of joints and a prolonged supply of HA. Most recently, Cao *et al.* (2019) developed a reversible peptide–PNIPAm hydrogel for controlled drug delivery purposes.[212] They used the conformational change of PNIPAm above their LCST as cross-links to connect different peptide nanofibrils to a 3D gel network. The transition temperature for a mixture of PNIPAm and a model peptide I_3K from sol to gel was measured at 33 °C. The loaded it with a antibacterial peptide ($G(IIKK)_3I\text{-}NH_2$), showing linear drug release over time.

5.3. Biomedical application of other thermoresponsive polymers

In Section 3, besides PNIPAm, poloxamers, elastin-like polypeptides and poly(*N*-vinylcaprolactam) have been presented. Since they are primarily not used as surface coatings, this section will give a short overview on how they are used and applied as materials for controlled drug delivery, as stimuli-responsive implants and in regenerative medicine, including tissue engineering.

Poloxamers like Pluronics® are not commonly used to fabricate surfaces for cell sheet engineering, but are rather used as a bulk material with thermoresponsive properties. For example, the group of Cohn *et al*. has investigated Pluronics for several years and published numerous articles about their application as thermoresponsive implant materials.[81,213–217] They focused mainly on the development of drug delivery systems and injectable/self-expanding materials. Using the thermo-reversible sol–gel-behaviour of Pluronic solutions, they could achieve gelation of injectable Pluronic inks inside the human body, using them as biomaterial or tissue engineering component. They successfully crosslinked Pluronics F127 dimethacrylate *in situ*, fabricating tubes with promising mechanical properties, which enables the construction of robust macroscopic structures for the use as implant biomaterials.[218] Furthermore, they fabricated and tested gels as drug carriers, which showed favourable release kinetics[81] for potential clinical applications. On the basis of Pluronics F127, they fabricated a temperature- and pH-responsive hydrogel to manufacture responsive 3D structures for the use as biomaterial.[215] They allow the fabrication of complex 3D structures that can change in space by adjusting temperature and pH (*e.g.* using temperature differences *in vitro vs. in vivo*).

Other research groups investigated the use of poloxamers in scaffold construction. Hospodiuk *et al.* described in their paper the use of Pluronics for extrusion-based biofabrication.[219] The thermoresponsive properties could be exploited to gain control over the extrusion process. Pluronics can be kept below their gelation temperature as liquid ink, which allows easy handling and incorporation of *e.g.* cells or proteins. In combination with a nozzle heated above the transition temperature of the material, it can be extruded as gel to form stable 3D-structures.[220] Müller *et al.* (2015) presented a Pluronic-based bioink for 3D-printing purposes, characterizing their mechanical properties and biocompatibility using bovine chondrocytes.[221] Gioffredi *et al.* (2016) examined Pluronics F127 for the use as cell printing material for the fabrication of cell-laden scaffolds.[222] They used a printing cartridge that allows heating and filled it with Pluronic F127 solutions with a concentration of 25%. The solution temperature was 4 °C, cells were incorporated. Gelation was achieved by heating of the cartridge to 37 °C and stable scaffold constructs with macropores could be printed. They showed, that the use of Pluronics F127 solutions is feasible for printing of cell-laden 3D-scaffolds. Low temperatures and rather harsh conditions during the printing process did not hamper cell viability. They could demonstrate that Pluronics can not only be exploited as sacrificial material for 3D-printing, but also as cell carrier material for the construction of cell-laden tissue engineering scaffolds. Vandenhaute *et al.* (2014) presented in their work a comprehensive investigation of Pluronics modified with bismethacrylate (BMA).[223] They described mechanical and physico-chemical properties for several Pluronics-BMA combinations. Maazouz *et al.* (2017) used Pluronics in combination with a calcium phosphate cement paste. The thermosensitive nature of Pluronics allows the control of injectability of CPC pastes for clinical applications.[224] The reverse thermal gelation behaviour of the poloxamer allows for the *in vivo* gelation at 37 °C, which is favourable for the CPC paste to maintain an initial mechanical stability until the CPC is set. Recently, a review focusing on application of Pluronics in drug and gene delivery was published by Rey-Rico and Cucchiarini.[225] Recently, Khan *et al.* (2019) tested poloxamers gels as depots for transdermal drug delivery.[226] The depots should form transdermal following microneedle application. Once the skin has been penetrated with the needles, the micropores are filled with poloxamer forming gels at 32 °C, delivering drugs *in situ*. They could show that the sol–gel transition of poloxamers is suitable for *in situ* formation of depots, allowing the controlled transdermal delivery of pharmaceutical agents after microneedle application. In summary, it is apparent that Pluronics represent a versatile class of material, applicable in the biomedical field and regenerative medicine. The sol–gel-transition in the range of body temperature holds potential for several applications, *e.g.* exploiting the injectability below body temperature for biomaterial injection, and following gelation *in situ*. Additionally, the thermal gelation can be exploited for 3D-printing, *e.g.* also in combination with cells at physiological relevant temperatures. Russo and Villa (2019) most recently published a review on poloxamers hydrogels in biomedical applications, presenting several examples for the use of poloxamers in the biomedical field.[227]

Besides Pluronics, elastin-like polypeptides attracted attention during the last years.[228–231] As mentioned before, they show an Inverse Temperature Transition (ITT), similar to the LCST behaviour of PNIPAm. At a certain temperature, they start to re-arrange in a self-structured manner, aggregate and become insoluble, forming fibrils and coacervates. This effect can be used for biomedical applications.[232] For tissue engineering purposes, Betre *et al.* (2006) exploited the good biocompatibility and bioactivity of ELP solutions and their temperature-induced sol–gel-transition.[233] They showed, that chondrogenic differentiation of human-derived adipose stem cells was induced and facilitated, without the use of chondrogenic supplements (*e.g.* growth factors like TGF-β). Embedding the cells in ELP solution and subsequent heating leads to aggregation of ELP chains, forming a viscous cell-coacervate mixture. The viscous fluid can be injected *in vivo*, allowing *in situ* scaffold formation. This allows precise structural and biological support in areas, where it is needed. However, ELPs do not form hydrogels when prepared in this manner and might not provide enough structural stability for applications in areas where high mechanical stability is required.[232,233] Bessa *et al.* (2010) used the self-assembly of elastin-like polypeptide particles for the delivery of bone morphogenic proteins (BMP).[234] They produced spherical nanoparticles (average diameter of 115 nm), loading them with BMP-2 and BMP-14 during particle preparation. Studying the release kinetics of the bone growth factors, they conclude that these loaded nanoparticles were able to deliver the BMP in a bioactive way, leading to enhanced mineralization. The release kinetics of these nanoparticles might facilitate bone formation *in vivo*. Furthermore, ELP loaded structures were used as depots to develop new therapeutic tools to treat cancer. Temperature triggered ELP depots could be locally applied in tumours, minimizing systemic toxicity of anti-cancer drugs. ELP solutions loaded with anti-tumour agents were injected into tumours, forming depots *in situ* because of their temperature-induced coacervation at body temperature. Loaded with radionuclides, the significantly facilitated tumour regression.[231] These kinds of depots have also been tested for the application in diabetes.[235] Glucagon-like peptide 1 (GLP-1), showing promise for the

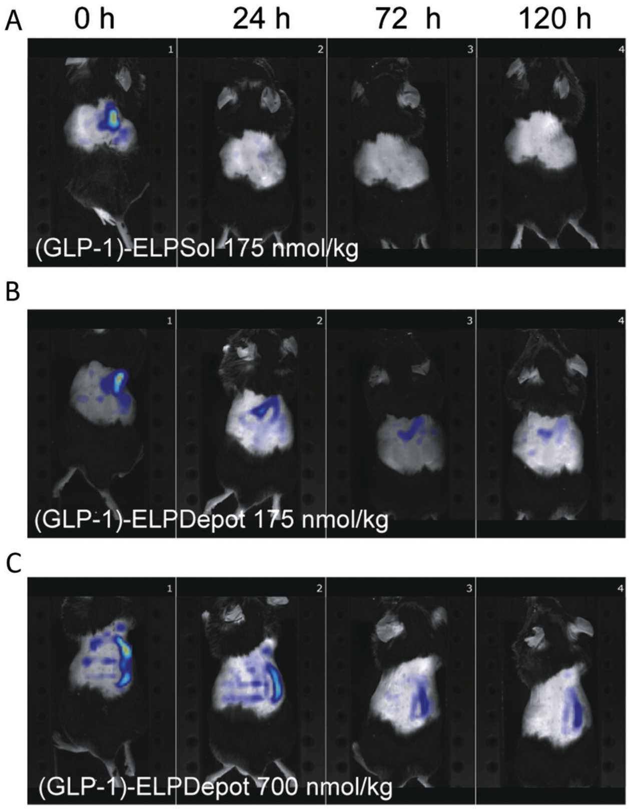
A
0 h
24 h
72 h
120 h
1
2
3
4
(GLP-1)-ELPSol 175 nmol/kg
B
1
2
3
4
(GLP-1)-ELPDepot 175 nmol/kg
C
1
2
3
4
(GLP-1)-ELPDepot 700 nmol/kg

Fig. 10 GLP-1 ELP depots (B and C, loaded with different GLP concentrations) showed a prolonged presence of the GLP-1 at the subcutaneous site of injection inside the mice over the course of 5 days (120 h). High concentrated GLP-1 ELP depots successfully reduced the fed glucose level over the course of 5 days. Adapted from Amiram *et al.* (2013).[235] Copyright©2013 Elsevier B.V. All rights reserved.

treatment of diabetes type II, has been combined with ELPs. Normally, GLP-1 is rapidly degraded in the organism and needs to be frequently administered to achieve a therapeutic effect. By tuning the transition temperature of ELPs below body temperature, subcutaneous depots loaded with GLP-1 could be formed. These depots, tested in mice, showed a continuous release of GLP-1 over the course of 5 days. The fusion with ELP provides a long-circulating carrier for the administration of GLP-1 inside the body. Fig. 10 shows the enhanced storage capability of GLP-1 when combined with ELP. For both concentrations of (GLP-1)-ELP depots, the GLP shows a prolonged activity in the subcutaneous area. Further applications of ELP in drug delivery are reviewed in more detail by MacEwan *et al.* (2014).[231] A more recent work involving ELPs as delivery systems has been published by Pal *et al.* (2019).[236] They loaded collagen–ELP hydrogel blends with doxycycline and recombinant human bone morphogenetic protein-2 (rhBMP-2) for bone regeneration. The drug-loaded hydrogels showed promising mechanical stability, a three-dimensional open pore structure and attachment and differentiation of human adipose stem cells (hASC), combined with a antibacterial bioactive behaviour. Overall, they state that the collagen–ELP hydrogels loaded with drugs are facilitating bone regeneration. The thermoresponsive behaviour of ELPs would provide the hydrogel with a sustained and prolonged rhBMP-2 release.

For surface modification, Costa *et al.* (2018) described in their recent publication, that elastin-like polypeptides could be used to form polyelectrolyte multilayer systems by combining them with chitosan.[237] Therefore, chitosan and ELPs were layered onto a substrate in an alternating process, whereas opposite charge of the polymers allowed a stacking of several layers. Physical characterization, in form of quartz crystal microbalance, water contact angle and atomic force microscopy, was performed showing the success of the layer-by-layer process and the thermoresponsive properties. Biocompatibility was tested using SaOS osteoblast-like cells. On PEM ending in ELP layer, cells showed adhesion and activity. However, these layers have a transition temperature at 50 °C, changing from hydrophobic state below to a hydrophilic state above it. This is not suitable for cell sheet engineering, but nevertheless, possess these layers potential for biomedical applications and use in regenerative medicine, *e.g.* as coatings to enhance cellular adhesion or to carry biologically active molecules. Despanie *et al.* (2016) comprehensively reviewed the state-of-the-art of elastin-like polypeptides with the perspective of biomedical applications, on which we expressly refer for detailed information on ELP.[238]

In recent years, the interest in PNVCL as synthetic polymer for biomedical applications has grown rapidly. There are several biomedical applications for PNVCL, ranging from entrapment of biomolecules and cells to drug delivery and tissue engineering. Cortez-Lemus *et al.* (2016) comprehensively described the manifold applications in their review on PNVCL.[115] Indeed, PNVCL was applied to modify surfaces for the use in cell sheet engineering applications. Lim *et al.* (2007) successfully fabricated surfaces consisting of a PNIPAm-PNVCL co-polymer and reported cell detachment from them in a thermoresponsive manner.[239] They used electron irradiation to fix the thermoresponsive co-polymer onto cell culture polystyrene dishes. Furthermore, Lee *et al.* (2013) showed, that they could retrieve cell sheets from a PNVCL-modified surface by simply lowering the temperature.[240] They used initiated chemical vapor deposition for the fabrication of the thermoresponsive surface. More recently, Sala *et al.* (2017) published a work showing promising results for PNVCL hydrogels as injectables for cartilage tissue engineering.[241] They embedded chondrocytes and mesenchymal stem cells in such hydrogels and were able to inject them into rats showing that formation of the hydrogels was triggered by increased temperature *in situ*. They could also find formation of cartilage ECM. Indulekha *et al.* (2016) investigated chitosan-PNVCL gels as transdermal drug release systems, which possess a LCST at 35 °C.[242] Prepared gels were characterized physical and biological, *i.e.* performing swelling, drug release and biocompatibility studies. Gels were loaded with two drugs: acetamidophenol and etoricoxib. They showed, that drug permeation could be triggered by increasing temperature to 39 °C (above LCST). *In vivo* skin irritation test showed good biocompatibility of the transdermal drug delivery system. This system shows promising results for the use as temperature triggered, on-demand drug delivery system. More recently experiments on PNVCL gels have been conducted by the group of Macchione *et al.* (2019).[243] They synthesized PNVCL nanogels (NG) *via* thermo-precipitation in aqueous solutions and free radical polymerization. One of their NGs, namely PVCL80$_4$NG, with a VCL concentration of 80 mg and 4% crosslinking agent (*N*,*N*′-methylenebisacrylamide) collapsed with increasing temperature. This is quite favorable, since it has a nanometric size after collapse at physiological temperatures, making it suitable for biomedical applications. Furthermore, the biocompatibility and antiviral effect against HIV-1 infections has been demonstrated. This is the first NG with *in vitro* inhibitory effect against R5-HIV-1, making the PNVCL-NGs a potential candidate for HIV-1 microbicide administration.

6. Conclusions

The review of literature performed here shows that poly(*N*-isopropylacrylamide) is the most researched and favoured thermoresponsive polymer. Over the last two decades and beyond, especially the group of Okano extensively studied PNIPAm, specifically as material for thermoresponsive surfaces. They limited their research on thermoresponsive PNIPAm-surfaces prepared by electron beam irradiation. Cell culture dishes of this kind are by now commercially available. Several publications and clinical studies were presented, showing successful cell sheet engineering and applications in tissue engineering that have been summarized in Table 2. Some tissue cell sheets grown on PNIPAm-surfaces are already clinically applied as tissue substitutes, *e.g.* as cornea replacement. However, this method presents certain limitations. In addition to the expensive equipment needed for electron beam irradiation, cells only grow and detach in a small range of thickness of the prepared PNIPAm coatings. Furthermore, cell adhesion on these surfaces is inferior to conventional cell culture polystyrene (PS) dishes. This led researchers to look for alternative surface preparation methods, of which have been presented in this review. However, they are not as extensively studied, especially with focus on clinical applications. More recently, the layer-by-layer technique has emerged as potential surface modification method, fabricating polyelectrolyte multilayer films that allow the precise control of thickness of surface coatings. Charged PNIPAm-*co*-polymers or grafting of PNIPAm to polyelectrolytes must be used for multilayer fabrication. A combination of the thermosensitive behaviour of PNIPAm with more biocompatible and bioactive polymers is conceivable. However, in comparison to the surfaces prepared by Okano, this is a relatively new technique applied for thermoresponsive surfaces and needs further research. One further approach of tuning PNIPAm properties is co-polymerization. It has been used to create derivatives and co-polymers with different properties, *e.g.* changes of the LCST. This has been used to create surface with different adhesion kinetics and more often for the creation of PNIPAm-based drug delivery systems. The wide variety of co-polymers makes PNIPAm very attractive for biomaterial research and future clinical applications.

Further on, we also highlighted the application of other thermoresponsive polymers. Pluronics, elastin-like polypeptides and PNVCL are less important for the fabrication of cell sheets and rather used in drug delivery and tissue engineering, in particular 3D-printing. Pluronics represents a versatile material group, which allow adjustment of their properties by changes

Table 2 Summary of studies and clinical trials of different cell sheets for different tissues

Material	Tissue	Cell sheets	Model	Ref.
Poly(*N*-isopropylacrylamide) PNIPAm	Cornea	Corneal epithelial	Rabbit	161 and 162
		Corneal endothelial	—	200
	Esophagus	Mucosal epithelial	Human/clinical trial	164
			Beagle dog	165
		Autologous epidermal	Porcine	167
	Middle ear	Nasal mucosal epithelial	Rabbit	168
		Autologous nasal mucosal epithelial	Human/clinical trial	169 and 170
	Periodontum	Periodontal ligament	Beagle dog	171
		Multi-layered periodontal ligament	Beagle dog	172
		Multipotent mesenchymal stromal cells	Beagle dog	175
	Cartilage	Chondrocyte sheets	Minipig	176
				177
			Rat	178
			Human	179
	Lung	Lung and skin fibroblasts	Rat	180
		Skin fibroblasts	Porcine	181
	Wounds	Fibroblasts	Rat	182
	—	Epithelial keratinocytes	Rat	184
	—	Monolayered islet cells	Rat	185
	—	Islet cell	Mice	187
	—	Allogenic adipose-derived stem cells	Rat	188
	Bone	Multipotent mesenchymal stromal cells	Rat	189
	Liver	Hepatocyte	—	190 and 191
		Hepatocyte	Mice	192
	Myocardium	Skeletal myoblast	Rat	196
	—	Heterotypic cell sheets		199

in chemical composition. They show reverse thermal gelation behaviour, which means they form gels above a certain transition temperature. Since this can be tailored to specific needs, applications in form of injectable materials (loaded with growth factors or drugs) that form a viscous gel inside the body because of the body temperature are conceivable. This gelation holds them in place, releasing drugs or providing structural stability *in situ*. This property could also be beneficial for 3D-printing. It allows an easy extrusion of a low viscous material by inducing gelation with a heated extruder tip. This makes handling of the material easier, furthermore enabling loading of the printing materials (*e.g.*, with drugs, cells, biomolecules). Also, elastin-like polypeptides have several advantages. Since they are of biological origin, they possess excellent biocompatibility. Their coacervation behaviour above a certain temperature makes them favourable for drug delivery inside the human body, especially for *in situ* drug release applications. In combination with other polymers, they can also be used in form of a surface coating. Unfortunately, the transition temperature is quite high and not suitable for cell sheet engineering.

Concerning PNVCL, research has shown its similarities to PNIPAm, preparing cell culture dishes in a similar fashion. They allow viable cell sheet recovery, presenting an alternative to PNIPAm, with slightly enhanced biocompatibility. Nevertheless, fabrication of these surfaces presents similar limitations as for PNIPAm. However, PNVCL shows promising results as injectable hydrogel for tissue engineering, in particular for cartilage. The thermoresponsive behaviour was further exploited for drug delivery applications, triggered by external stimuli.

Overall, PNIPAm is still the most studied thermoresponsive material and the ''gold-standard'' in cell sheet engineering. The aforementioned alternative materials are suitable for versatile applications, ranging from drug delivery to tissue and cell sheet engineering. Especially in the area of 3D-printing to fabricate tissue engineering scaffolds, the thermal gelation properties of materials like Pluronics and ELPs can be exploited. In contrast to cell sheet engineering, which is only applicable for tissue with low ECM amount, 3D-printing of scaffolds allows the fabrication of replacements for ECM-rich tissue. Concerning the fabrication of thermoresponsive surfaces, alternatives to electron beam irradiation were presented and especially the layer-by-layer fabrication of thermoresponsive polyelectrolyte multilayers seems to emerge as a promising method to create PNIPAm coatings of required thickness and corresponding cell adhesion properties, which deserves further investigations.

Conflicts of interest

There are no conflicts of interest to declare.

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