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## Effects of physicochemical properties of polyacrylamide (PAA) and (polydimethylsiloxane) PDMS on cardiac cell behavior

Karim Daliri,<sup>a</sup> Kurt Pfannkuche \*<sup>abcd</sup> and Bora Garipcan\*<sup>e</sup>

*In vitro* cell culture is commonly applied in laboratories around the world. Cultured cells are either of primary origin or established cell lines. Such transformed cell lines are increasingly replaced by pluripotent stem cell derived organotypic cells with more physiological properties. The quality of the culture conditions and matrix environment is of considerable importance in this regard. In fact, mechanical cues of the extracellular matrix have substantial effects on the cellular physiology. This is especially true if contractile cells such as cardiomyocytes are cultured. Therefore, elastic biomaterials have been introduced as scaffolds in 2D and 3D culture models for different cell types, cardiac cells among them. In this review, key aspects of cell–matrix interaction are highlighted with focus on cardiomyocytes and chemical properties as well as strengths and potential pitfalls in using two commonly applied polymers for soft matrix engineering, polyacrylamide (PAA) and polydimethylsiloxane (PDMS) are discussed.

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As in other organs of the human body, the physiological function and organogenesis of the heart are strongly influenced

by cellular adhesion, mechanosensing, and signaling, which are mediated by integrins. Members of this superfamily of cell adhesion receptors play key roles in cell adhesion, extracellular matrix (ECM) organization, survival, and proliferation. Twenty-four possible combinations of alpha–beta heterodimers deriving from a minimum of 18 alpha and 8 beta subunits of integrins have been identified in humans to date.<sup>1</sup>

The simultaneous interaction of many integrin receptors with ECM networks enables cells to acquire chemical and mechanical cues from the surrounding microenvironment.

<sup>a</sup> Institute for Neurophysiology, University of Cologne, Medical Faculty, Robert Koch Str. 39, 50931 Cologne, Germany. E-mail: Kurt.Pfannkuche@uni-koeln.de

<sup>b</sup> Department for Pediatric Cardiology, University Hospital Cologne, Cologne, Germany

<sup>c</sup> Marga-and-Walter-Boll Laboratory for Cardiac Tissue Engineering, University of Cologne, Germany

<sup>d</sup> Center for Molecular Medicine, University of Cologne, Germany

<sup>e</sup> Institute of Biomedical Engineering, Bogazici University, Cengelkoy, 34684, Istanbul, Turkey. E-mail: bora.garipcan@boun.edu.tr



**Karim Daliri**

Karim Daliri is a PhD candidate on stem cell biology and tissue engineering, Institute of Neurophysiology, Cologne University, Cologne, Germany. After obtaining BSc/MSc in Biomedical Sciences and several years of gaining practical experience, he joins Dr. Kurt Pfannkuche's research team in Cologne to continue his research career on stem cell biology, tissue engineering and molecular biology. His research interests and efforts are mainly regenerative medicine, genetics, nanotechnology and biomaterials.



**Kurt Pfannkuche**

Dr Kurt Pfannkuche studied biology at the University of Cologne and received his doctorate from the medical faculty in 2009. His main research interests are pluripotent stem cells and the cardiovascular system. His research focuses on regenerative medicine and issues of cardiac tissue engineering. Information about Pfannkuche's research group can be found at [www.cardiac-tissue-engineering.eu](http://www.cardiac-tissue-engineering.eu).



These environmental cues are converted into intracellular signals with a wide range of effects such as differentiation, growth, and energy production. As a result, changes in the microenvironment influence cell phenotype and its fate.<sup>2</sup>

The term integrin adhesion complexes (IACs) is used to describe a range of linker structures connecting different ECM proteins with each cell. These complexes are divided into focal complexes, focal adhesions, and fibrillar adhesions and contain a multitude of highly dynamic components that communicate through hundreds of interactions (Fig. 1).<sup>3</sup>

Considerable work has focused on the roles of specific constituents of integrin-mediated adhesions, generally termed the “integrin adhesome”, in different pathological conditions.<sup>4,5</sup> Mutations in specific components of the adhesome in different human diseases have received special attention, and both experimental as well as *in silico* methods have provided new perspectives on the molecular mechanisms that cause these pathological conditions, with a view to translation to clinical contexts.<sup>6</sup> For example, in the cardiovascular system, antagonists of  $\alpha$ IIb $\beta$ 3 integrin have been used as an anticoagulant drug in millions of patients.<sup>7</sup>

The main sources of extracellular mechanical signals are other cells, fluid flow, gravity, and polymeric scaffolds in tissue engineering applications.<sup>8–10</sup> Cells respond within two minutes to substrates with similar surface topologies but different elastic moduli.<sup>11</sup>

Integrins act at the molecular level through signaling cascades, which are mediated by integrin-linked kinase (ILK), focal adhesion kinase (FAK), Src kinase, and Rho GTPases (including Rac1, RhoA, and CDC42). These pathways are interconnected; for example, inactivation of RhoA requires Src. As a result, the network output resulting from these signaling pathways can influence the balance between stabilization or remodeling of adherent junctions, and can regulate actin assembly and actomyosin contractility.<sup>12–14</sup> Moreover, cellular phenotypes such as cell growth can be influenced by mechanical forces through integrins (Fig. 2).<sup>15</sup>

Interestingly, most of the intermediate biomolecules noted above, such as GTPase family members and Src, act as molecular crosslinks between integrins and cadherins.<sup>17,18</sup> Cadherins mediate cell–cell adhesion by forming adherens junctions, and it has been suggested that cadherins and integrins must communicate effectively to mediate cellular interactions that are necessary for appropriate development.<sup>19</sup> For example, during zebrafish development, while integrins are in their inactive conformation status,  $\alpha$ 5 integrins are physically associated with each other on adjacent cells, and N-cadherin stabilizes the complex of inactive  $\alpha$ 5 integrins.<sup>19,20</sup>

Cadherins are a family of mechanosensitive adhesion proteins and expression of specific isoforms of cadherins have an impact on the cellular phenotype.<sup>21</sup>  $\beta$ -Catenin is present in adherens junctions (multiprotein scaffolds that couple intercellular contacts with the cytoskeleton) and involved in crosstalk between cadherins, canonical Wnt and transforming growth factor beta-1 (TGF- $\beta$ 1) signaling. Molecular signaling or mechanical forces that disrupt cadherin–cadherin interactions between cells result in release of  $\beta$ -catenin into the cytoplasm where it acts with growth factors of the signaling network.<sup>22,23</sup>

## Integrin-mediated mechanotransduction in cardiac tissue

Cells usually change their structure and function corresponding to mechanical characteristics of the surrounding matrix or cell culture substrates. Therefore, controlling the mechanical features of cell culture substrates crucially depends on cell type and the experimental design. Artificial tissues need to be adapted to the cell- and tissue-specific Young's moduli and potentially viscoelastic properties of the tissue should not be neglected.

Cells detect the stiffness of the extracellular environment *via* integrin-based signalling.<sup>24</sup> Integrins play a central role directly as a mechano-transducer to transmit the forces to other related factors or indirectly as a transitional element on pathways triggered by other receptors. It has been widely studied that the effects of mechanical forces on organ physiology are extensive and this is especially important for the cardiovascular system.<sup>25</sup>

Solid tissues are generally flexible and elastic on a macro-scale and small changes can be described by the Young's modulus ( $E$ : a measure of the stiffness of an elastic material measured in Pascal). Tissues have a broad range of elasticities *i.e.* the most flexible is the brain (<1 kPa) and mineralized bone is considered the stiffest.<sup>26,27</sup> It should be also pointed out that mechanical properties can differ based upon the resolution with which they are measured. The macroscale elasticity of a tissue reflects its structure on a larger scale and this can be different from the elasticity that a cell faces on a micron scale or smaller.

Numerous ECM proteins can be fabricated on gels with a measurable elasticity. For example, Matrigel which is a combination of secreted proteins (also contains a large amount of growth factors) originated from mouse sarcoma cells. Although



**Bora Garipcan**

*Dr Garipcan received his BSc (1999) from Hacettepe University, Department of Chemistry, and his PhD (2008) in Bioengineering from Hacettepe University, Ankara, Turkey. His main research area is, Biomimetic and Bioinspired Biomaterials. He is a member of Boğaziçi University, Institute of Biomedical Engineering, Istanbul, Turkey, since July 2011. Garipcan's research laBORAtory focuses on surface engineering of biomaterial surfaces (such as Ti, Si, Au, biodegradable polymers, and elastomers) by changing surface properties (stiffness, topography, chemistry and biochemistry) for controlled and directed cellular behavior (adhesion, proliferation, and differentiation).*



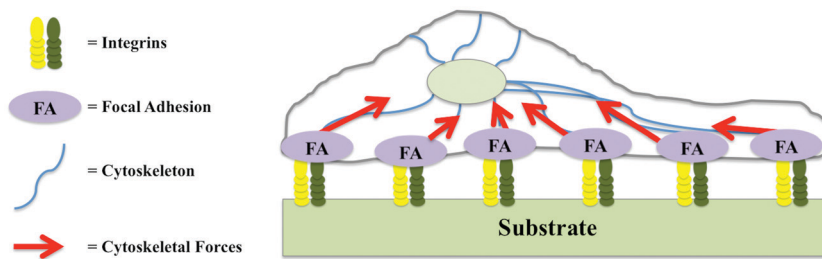


Fig. 1 Dynamic components of integrin adhesion complexes.

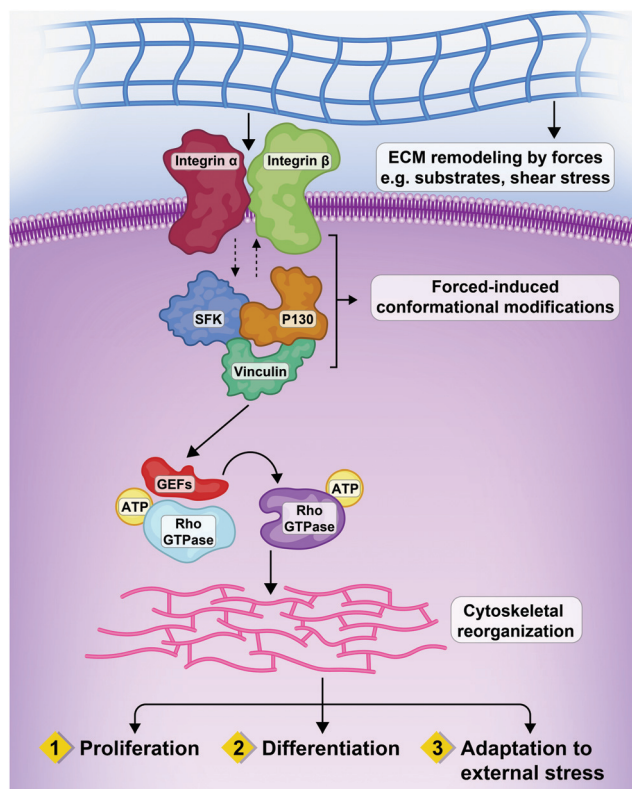


Fig. 2 Interactions between extracellular forces, matrix remodeling and integrins resulting in three patterns of cellular response. Mechanical interactions of cells with the ECM by heterodimeric transmembrane receptors called integrins, that are physically connected to cytoskeleton. Mechanical cues from the micro-environment of the cells can impact on cell proliferation, differentiation and adaptation through the Rho GTPases and relevant effectors.<sup>16</sup>

these gels can be considered a suitable substrate for investigating of the cell behavior, modifying gels to control stiffness such as density changes and crosslinking can alter the density of the ligands. Indeed, natural materials such as Matrigel have a complex composition that makes it difficult to distinguish between mechanical and biochemical effects.<sup>28</sup>

Synthetic hydrogels can be simplified with more tangible biological effects. For example, PAA gel coated with a non-fibrillar collagen ligand with a specific density is a commonly used system. And its flexibility can be simply manipulated by varying the cross-linking and/or density of polyacrylamide. Indeed, these systems

have been widely used to mimic tissue microenvironments and to investigate cell responses.<sup>29</sup>

One of the main mechanosensitive components inside muscle cells are called the costamers (Cstms) which are located beneath the sarcolemma (specialized membrane that surrounds striated muscle fiber cells) and is physically attached to the ECM by transmembrane integrins.<sup>30</sup> Major function of Cstms is transmission of contractile forces bilaterally from sarcomeres (contractile unit of a striated muscle fiber) to sarcolemma and then to the ECM and finally to neighboring muscle fibers.<sup>31</sup>

Costamers are composed of three types of multi-protein complexes which are (1) dystrophin–dystroglycan complex, (2) spectrin–ankyrin cytoskeleton complex, and (3) integrin receptor complex.<sup>32</sup>

ECM–integrin–costameric protein complex is a mechanosensory apparatus. In this complex, the role of the Cstms in addition to attachment is sensing of mechanical forces such as active tension resulting from development of cardiomyocytes – in partnership with focal adhesive complexes – and converting them into biochemical signals leading to sarcomeric assembly leading to gene expression modifications. This form of signaling is known as “outside-in” and represents the major mechanism responsible for adaptive cardiomyocyte growth in response to dynamic loads. It is also noteworthy to mention that stretch-induced deformation of cardiomyocyte integrins initiates the induction and activation of various kinases such as FAK, Src, and Rho to the cytoplasmic side of the focal adhesion complex where they participate in signaling to the nucleus and other organelles.<sup>33,34</sup>

Integrins are a family of 24 transmembrane  $\alpha\beta$  heterodimers – at least 18  $\alpha$  and eight  $\beta$  subunits are known in humans – each of them specific to a particular set of ligands in the ECM.<sup>35,36</sup> Through a process called integrin activation, which involves conformational changes in the integrin ectodomain, integrin-mediated adhesion commences, causing a low-to-high affinity state shift for ligand binding.<sup>37</sup> In addition, responses to substrate stiffness depend heavily on the type of cell. Cardiomyocytes show a strong response, which is reflected as the intact or disrupted assembly of sarcomeres according to the shape of the cells.<sup>38</sup>

Aberrant integrin expression in the heart is assumed to be linked to severe pathologies. It was found that a specific integrin  $\beta 1$  knockout in ventricular cardiomyocytes resulted in the inability of the murine heart to resist increased hemodynamic loading, accompanied by the development of cardiac fibrosis and dilated cardiomyopathy.<sup>39</sup>





Recently, Wang and colleagues found that integrin  $\beta 1$  is downregulated in heart tissues of patients with arrhythmogenic right ventricular cardiomyopathy; these authors were able to link low integrin  $\beta 1$  with defective calcium handling by ryanodine receptor type 2 (RyR2).<sup>40</sup> In a mouse model ( $\beta 1D$ ) with integrin-deficient cardiomyocytes, catecholamine-sensitive polymorphic ventricular tachycardia was seen – a finding that further supported the conclusion that impaired integrin signaling can result in cardiac arrhythmia.<sup>41</sup> Moreover, altered alpha integrin expression may result in cardiac fibrosis and hypertrophy, as demonstrated in murine models of integrin  $\alpha 11$  overexpression and deletion.<sup>42</sup>

## Cardiomyocytes and matrix elasticity

Cardiomyocytes are exposed to different mechanical cues including hemodynamic pressure of the blood, active stretching forces during contraction, and passive elasticity of the ECM. The elastic properties of the matrix have a marked impact on cardiomyocyte physiology. Embryonic cardiomyocytes show stable autonomous contractions on scaffolds with tissue-matched elasticity (11 kPa), but overstrain themselves on substrates with fibrotic scar-like rigidity.<sup>38</sup> In neonatal rat cardiomyocytes improved sarcomere alignment, maturation, and force generation were found on gels with a Young's modulus of 10 kPa, whereas on stiffer substrates, stress fibers appeared and sarcomere alignment was reduced.<sup>43</sup> Other reports showed evidence of sarcomere fracture in cardiomyocytes grown on gels with a Young's modulus of 30–35 kPa (Fig. 3).<sup>44,45</sup>

Stem cell-derived cardiomyocytes were successfully cultured on hydrogels with tissue-matched stiffness for up to 7 weeks without loss of quality.<sup>46</sup> In contrast, rigid polystyrene (PS) cell culture dishes triggered structural damage and eventually loss of contractility. The fact that long-term cultures of pluripotent stem cell-derived cardiomyocytes have often been obtained on PS can be explained by the observation that the cell layer partially lifts from the substrate during differentiation, and this does not contradict the conclusion that rigid substrates are deleterious to autonomously contracting

cells. One recent study deciphered the effect of substrate elasticities matching the properties of fetal, adult, and scarred myocardium on the transcriptome of cardiomyocytes, and found substantial reactivation of ECM genes in cardiomyocytes exposed to a fibrosis-like matrix.<sup>45</sup> These examples, and further evidence in the literature, led to the conclusion that matrix elasticity is important to maintain cultured cardiomyocytes in a physiological state.

The mechanisms underlying the ability of cardiomyocytes to sense passive elasticity in the matrix have long remained enigmatic. In 2018, Pandey and colleagues shed light on these mechanisms by showing that cardiomyocytes sense combinations of slow nonmuscular myosin contractions and contractions exerted by fast muscular myosin.<sup>47</sup> Intracellularly, mechanical force is converted into cyclic stretching of the adaptor protein talin, which links integrin receptors with the cytoskeleton. Cyclic talin stretching occurs when cells face physiological substrate elasticity, whereas continuous talin stretching is found in cells exposed to stiffer environments. The fact that cardiomyocytes responded to matrix elasticity provides the grounds to reconsider strategies for culturing cells on stiff PS surfaces.

To better investigate the physiology and pathophysiology of cardiomyocytes *in vitro*, no study is complete without considering chemical factors of the polymers involved and the cellular responses to them. The next part of this review will focus on polyacrylamide (PAA) and polydimethylsiloxane (PDMS), two polymers used frequently for *in vitro* studies. A recent PubMed search for either “PAA” and “PDMS” with all type of cells ended up with higher number of articles with PAA compared to PDMS. However, when cardiomyocytes used as a keyword articles with PDMS were higher than PAA (Fig. 4).

## Chemistry of polyacrylamide and its polymerization

Polyacrylamide [IUPAC poly(2-propenamide), hereafter PAM] is a polymer ( $-\text{CH}_2\text{CHCONH}_2-$ ) formed from acrylamide subunits.

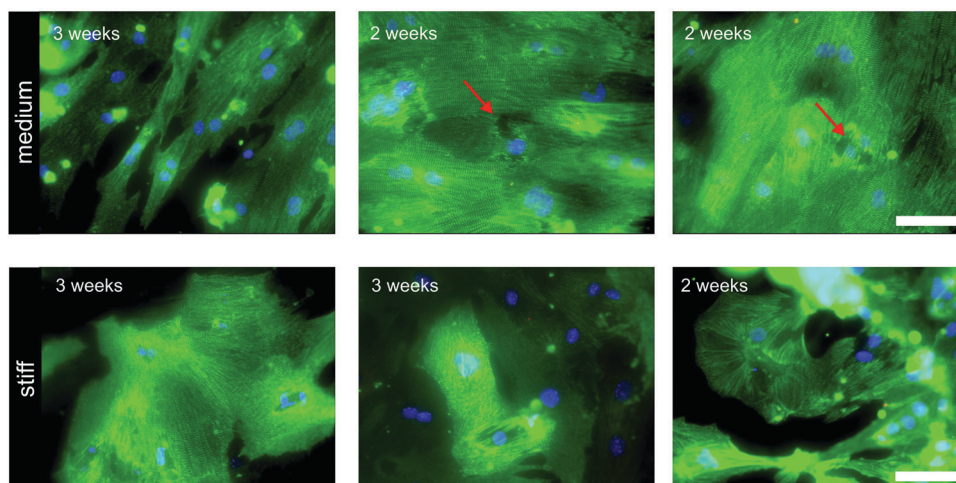


Fig. 3 Sarcomeric structure in murine induced pluripotent stem cell derived cardiomyocytes cultured for 2–3 weeks on medium (Young's modulus 35 kPa) and stiff hydrogels (Young's modulus 140 kPa). Immunocytochemical staining of z-discs was performed with antibodies against sarcomeric alpha actinin (green). Red arrows point to cells with sarcomere disruption. Scale bar: 100  $\mu\text{m}$ . Figure with permission from Heras-Bautista CO, N. Mikhael *et al.*<sup>45</sup>



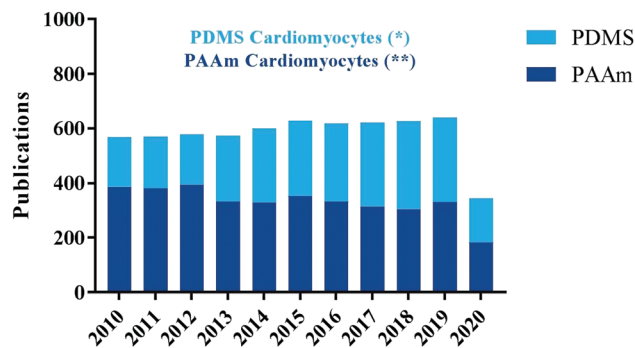


Fig. 4 Results from PubMed searches for either “PAA” and “PDMS” with all type of cells ended up with higher number of articles with PAA compared to PDMS. However, when cardiomyocytes used, as a keyword articles with PDMS (\*66) were higher than PAA (\*\*29) and doubled in number since 2010. Data retrieved on August 2020.

Polyacrylamide with only acrylamide monomers is nonionic; other monomers such as acrylate or 2-acrylamido-2-methylpropane sulfonate (AMPS) can be copolymerized at various percentages to form anionic PAM. Among the common co-monomers of cationic PAM are dimethyl diallyl ammonium, ethanaminium (*N,N,N*-trimethyl-2-((1-oxo-2-propenyl)oxy)) and 1,2-dimethyl-5-vinylpyridinium.<sup>48</sup>

Various methods have been used to synthesize polyacrylamides, such as solution, emulsion, and dispersion polymerization.<sup>49,50</sup> However, PAA gels are usually obtained through copolymerization

of acrylamide with a bifunctional monomer, or by radical polymerization of acrylamide followed by crosslinking (Fig. 5).<sup>51</sup> In general, the three integral components of PAM hydrogel preparations are the monomer, initiator, and crosslinker. Among the factors that influence acrylamide polymerization kinetics, the most important is monomer concentration.<sup>52</sup>

To prepare a PAA network structure, the initiator is used as a source of chemical species that reacts with a monomer to create an intermediate compound able to link successively with other monomers.<sup>56</sup> The type of initiator has a profound effect on the polymer product. When initiator concentration is increased, the number of active sites available to react with monomers also increases. Consequently, growing oligomers have better contact with monomers, and the possibility of termination reactions increases.<sup>57</sup>

Chemical polymerization is usually initiated with ammonium persulfate (APS), whereas photochemical polymerization is initiated with riboflavin (or riboflavin-5'-phosphate) as a nontoxic photoinitiator, or with a combination of riboflavin and APS.<sup>57</sup> It should be noted that when riboflavin is used, progress of the reaction is easily modified by using different initiator concentrations and light intensities.<sup>58</sup> In addition, initiation and polymerization can be catalyzed by tetramethylethylenediamine.<sup>59</sup> The free radicals generated from persulfate or riboflavin have oxidation and decomposition potential.<sup>58</sup> During copolymerization and crosslinking reactions, monomers are mixed with the multifunctional crosslinking agent, and polymerization is initiated

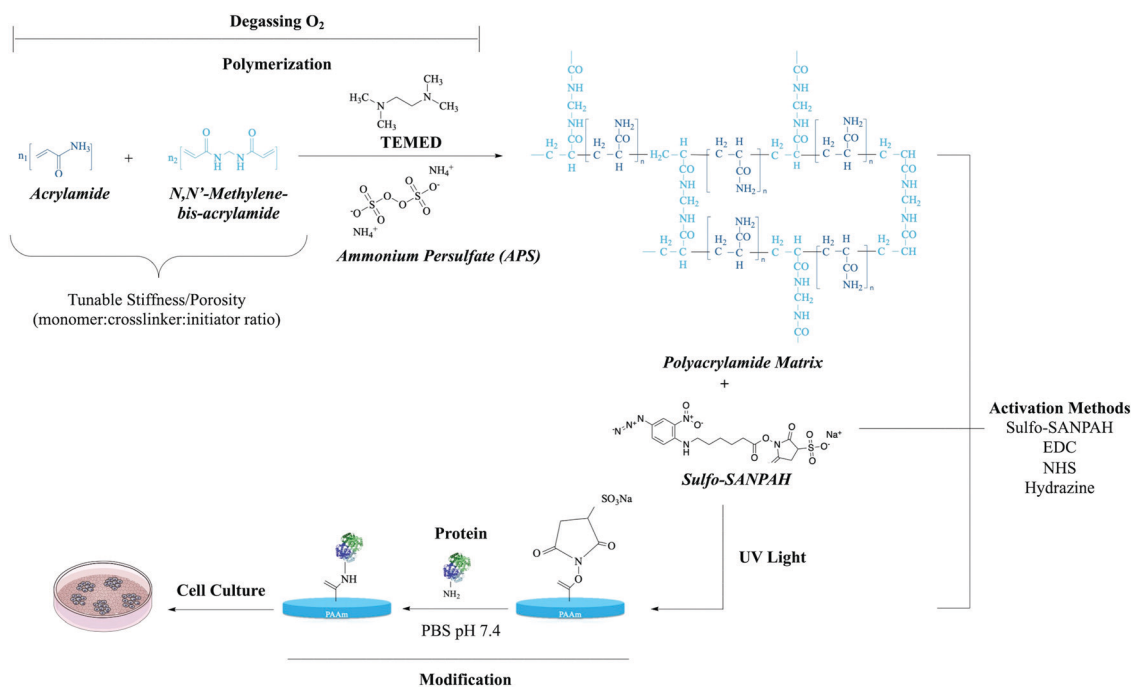


Fig. 5 The polymerization reaction of acrylamide.<sup>53</sup> *N,N*-Methylene-bis-acrylamide (BIS) acts as a cross-linker between acrylamide monomers. Chemical polymerization initiated by ammonium persulfate (APS) in the presence of tetramethylethylenediamine (TEMED). Stiffness and porosity can be tuned by total concentration of monomer and cross-linker. Sulfo-SANPAH is a heterobifunctional reagent generally used for the covalent conjugation of biomolecules to the PAA structure.<sup>29</sup> Carbodiimide (EDC),<sup>54</sup> NHS-acrylate<sup>29</sup> and hydrazine<sup>55</sup> are other conjugation methods to the PAA structure. Abbreviations: sulfo-SANPAH (sulfosuccinimidyl 6-(4-azido-2-nitrophenylamino)hexanoate); EDC(*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride); NHS-acrylate (*N*-hydroxysuccinimidyl acrylate).



thermally with ultraviolet (UV) irradiation or with a redox initiator system.<sup>59</sup>

The target gel can be synthesized as a simple linear-chain structure or crosslinked, typically with *N,N'*-methylenebisacrylamide as a crosslinker.<sup>60</sup> In the crosslinked form (the most favorable form) the presence of the monomer is markedly reduced. In other words, a soft insoluble gel forms in a highly water-absorbent milieu by hydration.<sup>61</sup> The extent of crosslinking affects the physical and chemical properties of the hydrogel. The parameters altered as a result of crosslinking are mainly elasticity, insolubility, increased glass transition strength and toughness, and transformation of thermoplastics into thermosets. These effects improve the mechanical properties of the hydrogel.<sup>62–64</sup>

The polymerization of acrylic acid with a water-soluble crosslinker, *e.g.*, 1% *N,N'* methylenebisacrylamide in an aqueous solution, is a straightforward process.<sup>65</sup> PAM hydrogels can also be crosslinked by gamma irradiation.<sup>66</sup>

Toxic compounds as crosslinking agents have adverse effects on the environment as well as undesirable reactions with the bioactive substances in the hydrogel matrix. However, these side effects can be prevented by using physical crosslinking, *e.g.* with radiation or an electron beam. The ability to control the amount of agent through dose manipulation has made radiation methods more suitable in terms of energy efficiency and the absence of undesirable residuals in the products.<sup>67,68</sup>

## Manipulating the surface properties of polyacrylamide

### Patterning of polyacrylamide gels

Thin layers of PAM can be used on a rigid substrate such as a cell culture plate or glass coverslip by coating the substrate with aminosilanes to favor adherence and immobilize the PAM hydrogel on the target surface. Because PAM hydrogel is bio-inert, lacking surface receptors or cell interaction sites, conjugating adhesive proteins to its surface is mandatory for cell attachment.<sup>69</sup>

Patterning PAM gels has been suggested as a key approach to optimize the creation of a favorable environment; however, hydrogel characteristics and the aqueous environment are two major obstacles to patterning.<sup>69,70</sup> The use of a reducing agent such as hydrazine hydrate to make PAM reactive to oxidized proteins is one suggested option to overcome these obstacles.<sup>55</sup>

To avoid the use of unstable chemicals such as reactive esters and expensive photoreactive agents such as sulfo-SANPAH for the surface functionalization of PAM hydrogels, a novel PAA hydrogel called hydroxy-polyacrylamide (hydroxy-PAM) has been suggested. Hydroxy-PAM remains stable and active for several weeks, can be mass-produced and easily microprinted, and has high affinity for biomolecules, and so can be used to evaluate the synergistic effects of conjugated ECM proteins on cellular functions. Moreover, hydroxy-PAM hydrogels can be used to quantitatively study the amount of contractile forces exerted by cells on their surrounding microenvironment. However, as in dozens of other microprinting-based

methods, the main limitation of hydroxy-PAM hydrogels for microprinting processes is the spatial resolution of protein microfeatures. In other words, on stiff hydrogels (>10 kPa), spatial resolution is on the micrometer scale and is determined by the resolution of the stamp, whereas on “soft” hydrogels, resolution is limited to some extent by surface deformation in the course of protein transfer. Hydroxy-PAM allows the immobilization of any type of ECM protein. Indeed, combining hydroxy-PAM hydrogels with microcontact printing can independently control the shape of single cells, matrix stiffness, and ECM protein density. These features make hydroxy-PAM hydrogels helpful to decipher key mechanisms of complex cellular and tissue processes related to the physicochemical properties of the cell microenvironment, such as wound healing, tissue homeostasis, and the pathogenesis of diseases such as fibrosis and cancer.<sup>71,72</sup>

One useful method of patterning is soft lithography (comprising a group of techniques), which has been shown to be an efficient approach to patterning for biomaterials. The term “soft lithography” indicates that these techniques fabricate or replicate structures by stamps made from an elastomeric or soft material, most notably PDMS; however, because of its less complicated microfabrication steps and lower oxygen permeability, parylene C is typically used for patterning in PAM hydrogels.<sup>73,74</sup>

Generally, there are two main strategies for patterning ECM on PAM gels. One is selective activation of the gels for covalent attachment of proteins to activated regions, such as direct surface functionalization by applying UV-reactive, sulfo-SANPAH crosslinkers or polymerizing *N*-hydroxyacrylamide to the hydrogel surface.<sup>72,75,76</sup> The other strategy is copolymerization of ECM proteins in the gels. Fibronectin (FN), laminin (LN) and collagen I are the cell adhesion molecules most commonly used for PAM gel patterning.<sup>69</sup> The first approach utilizes expensive functionalization reagents and depends on reagent quality and reaction time. These chemicals are unstable in aqueous media and in the presence of oxygen. However, some functionalization agents can be generated in the laboratory with basic equipment, and this reduces the costs of experiments significantly. A definite advantage of this functionalization approach is its stability for long-term culture experiments. The method of copolymerizing ECM proteins relies on patterning glass coverslips with protein and placing them in direct contact with the hydrogel during polymerization.<sup>77</sup> In this method, cells stably adhere to the hydrogel surface and ECM proteins are immobilized, but the mechanisms of protein immobilization, entanglement and chemical binding are not well understood. This approach has been used successfully to functionalize hydrogels with different ECM proteins.<sup>78,79</sup>

The photoresist lift-off patterning method was reported recently, and makes it possible to control the shape and size of epithelial cells. However, the application of the method is nontrivial.<sup>76</sup>

Techniques for patterning proteins on PAM are still frequently hampered by the lack of quantitative assessment, which is necessary in order to develop and compare protocols to reliably restrict cells to well-defined shapes. Due to the chemical flexibility of hydrogels and their wide range of



applications, the direct patterning of biocompatible gel structures is an approach that merits further investigation.

A final consideration is that the spatial resolution and accuracy of protein patterns directly impact cellular responses, and this is of particular importance in mechanobiological studies.<sup>80</sup>

### Plasma treatment

The formation of polymeric materials under the influence of plasma is called plasma polymerization.<sup>81</sup> PAM can be physically adsorbed onto a hydroxylated silicon surface to form a PAM film. These films can also be treated with nitrogen ( $N_2$ ) plasma, and peak intensity is detected at  $1214\text{ cm}^{-1}$  ( $NH_2$  stretch vibration), which confirms that  $N_2$  can graft to the PA surface in the process of  $N_2$  plasma treatment. Furthermore, surface tension is enhanced with increasing plasma grafting time. In the air, surface energy decreases rapidly in the initial phase and eventually reaches equilibrium. This indicates that some of the ions and alkyl radicals adsorbed on the PA surface can rapidly lose their activity. Indeed, the reason for increasing the surface tension of plasma-treated PAM films is to favor covalent binding of the amine groups to the PAM surface.<sup>82</sup> Treating the homopolymer surface with plasma can control the solid surface properties in a fast, simple, clean, and non-solvent-dependent manner. In addition, the surface properties of the polymers can be controlled by injecting different types of gases.<sup>83,84</sup> The penetration of cold plasma into the top nanometers of the polymer surface results in the formation of a fairly uniform surface with no influence on bulk properties.<sup>85</sup>

In addition, a broad range of compounds can be used as a monomer for plasma polymerization, providing a great diversity of possible surface modifications. Although a number of efforts have resulted in different applications for plasma technology, such as adhesion to composite materials, protective coatings, and plasma printing, polymerization remains a very complex process. Indeed, a number of parameters, including reactor design, power input, monomer flow rate, substrate temperature, chemical composition of the polymers, plasma gases, plasma pressure, and the treatment dose, may limit the application of plasma treatment methods.<sup>86–88</sup>

## Chemistry of polydimethylsiloxane

Polydimethylsiloxane belongs to the group of silicones which comprises silicon, carbon, hydrogen, and oxygen. PDMS is a three-dimensional silicone formed by the crosslinking of linear silicone molecules (Fig. 6). The crosslinking rate and extent of this reaction are significant factors that determine PDMS performance. Non-crosslinked PDMS may be almost liquid or semisolid.<sup>89</sup> The chemical formula of PDMS includes a flexible Si–O backbone and a repeating  $Si(CH_3)_2O$  unit. The molecular weight is basically determined by the number of repeating units, and as a result, many of the viscoelastic properties of the material can be defined based on the application of

interest. This polymer is also a relatively inexpensive, easy to mold, and very permeable to gases.<sup>90</sup>

Conventional PDMS synthesis usually begins with dichlorosilanes by hydrolysis and condensation, which yields cyclic and linear polymers. This method, however, leads to the synthesis of products with poor molecular weight control which cannot be utilized as model materials for particular purposes. For this reason, in order to better control the molecular parameters, this approach has gradually been replaced by ring opening polymerization of cyclic siloxanes. This process changes a specific cyclic siloxane monomer into a linear siloxane polymer by cleavage of the Si–O–Si bonds in the monomer ring, causing reformation of this bond in the polymer network.<sup>93</sup>

It should be noted that although PDMS has adjustable stiffness values, it does not promote protein adsorption or cell adhesion due to its hydrophilic nature.

## Manipulating the surface properties of polydimethylsiloxane

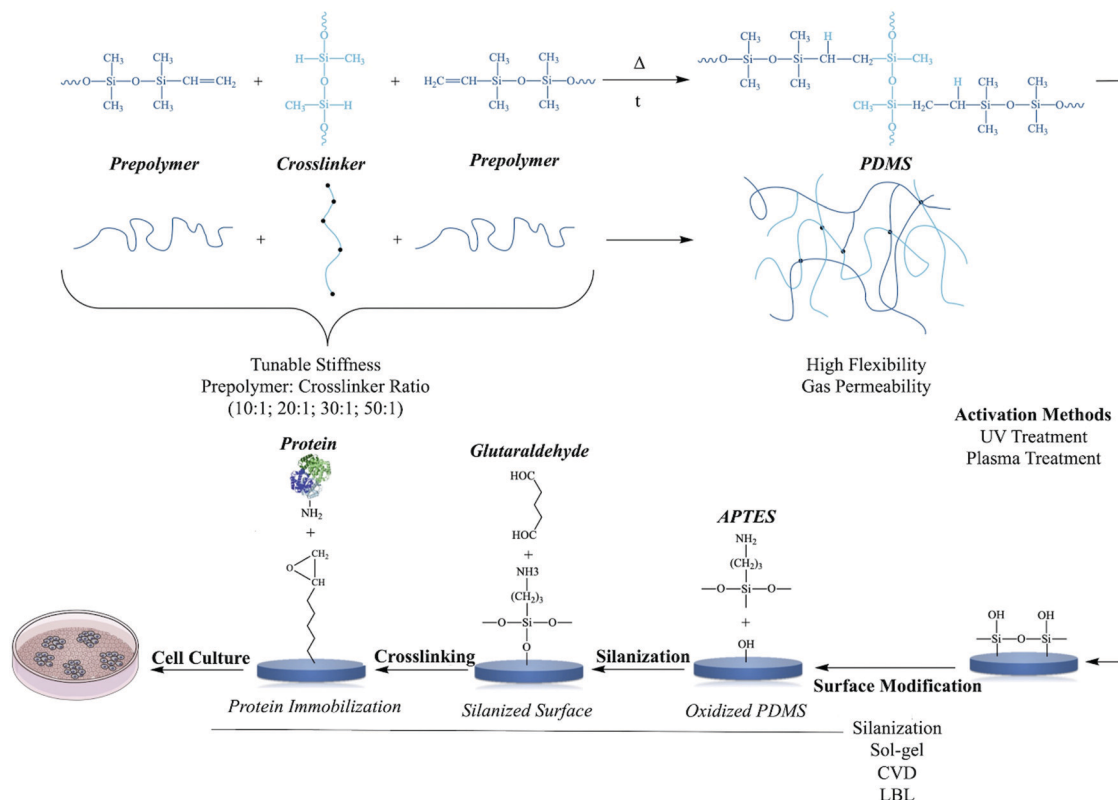
The initial interaction between biomaterials and cells is determined by chemical composition, surface energy, elastic and viscoelastic properties, and the topography and roughness of biomaterials. Indeed, cells can react to topographical features by changing their morphology and behaviors, such as attachment, migration, and gene expression.<sup>94</sup> Examples of topographical features of the target biomaterial that can trigger the cell response at the nano- (less than 100 nm), micro- (100 nm–100  $\mu\text{m}$ ), and macro-roughness (100  $\mu\text{m}$ –1 mm) scale are fibers, grooves, ridges, steps, pores, wells, and nodes. A major drawback of PDMS is its hydrophobicity and rapid hydrophobic recovery after surface hydrophilization. Therefore, it is mandatory to improve the surface biocompatibility of PDMS to facilitate long-term cell culture. Because surface wettability is a crucial factor that influences cell adhesion to the substrate, most studies have focused mainly on modifying the PDMS surface to decrease its hydrophobicity. This characteristic of the PDMS surface is easily modified by protein adsorption, plasma oxygenation, or arginylglycylaspartic acid (RGD) peptide conjugation, to create surfaces that promote cellular adhesion and biorecognition.<sup>95–98</sup>

Protein adsorption on the PDMS substrate has been widely used to facilitate cell adhesion because of its intrinsic biocompatibility with proteins, and its molecular recognition properties. However, maintenance of cell adhesions on these surfaces was transitory, and the cells were detached after reaching confluence or else aggregated to form loosely-bound cell clumps.<sup>99</sup>

Protein adhesion and aggregation during adsorption depends on the interaction between charged domains on the protein and the material surface. These interaction forces (*e.g.*, electrostatic, van der Waals, and hydrophobic) are usually weak and highly susceptible to protein leaching into the medium, so efforts have been made to induce strong, stable covalent linkages between the protein and the material surface.<sup>100,101</sup>







**Fig. 6** Polymerization reaction between prepolymer and cross-linker.<sup>91</sup> UV and plasma treatment are two commonly activation of PDMS surfaces to create hydroxyl (–OH) groups for the modification and the conjugation with molecules and biomolecules.<sup>92</sup> Silanization, sol–gel, chemical vapor deposition (CVD) and layer-by-layer are methods for the modification of PDMS polymer for further applications.<sup>92</sup> Silanization is given as a model modification of PDMS polymer with proteins for cell culture studies.

3-Aminopropyl triethoxy silane (APTES) and glutaraldehyde function as molecular spacers to minimize the direct, weak interactions of proteins with the PDMS surface and to overcome steric hindrance from the environs of the support two essential steps for stronger protein attachment.<sup>102</sup> The use of this modified PDMS surface to stabilize cell adhesion and support cell proliferation should study further due to its potential ability to extend the scope of research to include interactions of stable ECM protein-activated surfaces with adherent cells. Reducing PDMS hydrophobicity by oxygen plasma treatment can also improve cell adhesion, although the gains are often short-lived because of hydrophobic recovery.<sup>102</sup> Recent evidence showed that plasma treatment of PDMS resulted in consistent surface stiffening at depths of up to 1  $\mu\text{m}$ , whereas stiffness decreased exponentially at depths of 1 mm.<sup>103</sup>

Another technique to enhance cell adhesion is plasma etching followed by collagen adsorption to convert PDMS from hydrophobic to hydrophilic. The frequently used matrix protein collagen can accumulate on the surface *via* weak forces (*e.g.* electrostatic, hydrophobic, and van der Waals), and this can be followed by leaching of the collagen molecules into the solution and nonuniform collagen coating.<sup>104</sup>

One study used APTES and crosslinker glutaraldehyde (GA) chemistry to immobilize FN and collagen type 1 on PDMS and then evaluate the adhesion and viability of mesenchymal stem

cells (MSCs) on the prepared surfaces. Hydrophobicity of the original PDMS was significantly reduced. The adhesion of MSCs was mostly favorable when APTES and glutaraldehyde (APTES + GA) were used. In addition, the spreading area of MSCs was significantly higher on APTES + GA + C1 (collagen type 1) surfaces in comparison to other unmodified or modified PDMS surfaces with C1 adsorption, and there was no significant difference in MSC spreading area on unmodified or modified PDMS surfaces with FN adsorption. These findings indicate that the covalent surface chemical modification of PDMS with APTES + GA protein produced a more biocompatible platform for improved MSC adhesion and proliferation.<sup>102</sup>

Biocompatibility and cell differentiation on substrate surfaces can also be improved by applying both positively and negatively charged ions. The amount of positive charge on the surface of biomaterials can influence cell behavior. Several studies have shown that cell adhesion and proliferation can be modulated by surface charge density.<sup>104,105</sup> For example, as the charge density of hydrogels based on 2-hydroxyethyl methacrylate and 2-methacryloxyethyl trimethyl ammonium chloride copolymer increased, cell adhesion and proliferation improved markedly.<sup>105</sup>

Studies of polyethylene surfaces with various chargeable functional groups (–COOH, –CH<sub>2</sub>OH, –CONH<sub>2</sub>, and –CH<sub>2</sub>NH<sub>2</sub>) showed that cell adhesion, growth (in terms of the number of





cells attached), and spreading rate were optimized on polar and positively charged surfaces (amine group), whereas on negatively charged surfaces (carboxylic acid group), less growth was observed.<sup>106</sup>

Cell adhesion can also be modulated through protein adsorption, *e.g.* integrin binding on negative charged modified surfaces. Several studies have reported that surface charge along with wettability properties influence protein adsorption and cell adhesion.<sup>107</sup>

Wettability can be considered the main controlling parameter that influences cell behavior on smooth and rough surfaces, compared to the influence of polymer chemistry or the topography of superhydrophobic surfaces. Surface wettability can be easily modified by adjusting roughness.<sup>108,109</sup>

The molecules of PDMS have low surface energy, with a contact angle of  $\sim 110^\circ$ . By microstructuring the PDMS surface, contact angles significantly larger than  $110^\circ$  can be obtained. Surface morphology can also be modulated by changing the duration of exposure to UV or ozone radiation. For example, UV irradiation has been used to modify PDMS substrate stiffness from 0.24 MPa to 1.67 MPa or between 0.75 MPa and 2.92 MPa.<sup>110,111</sup>

Other research found that a micropore size of 1–2 mm is the most suitable for cell adhesion, and that the effect of pore size on cell adhesion is greater than the effect of surface hydrophilicity or hydrophobicity.<sup>112</sup> Additionally, PDMS surface roughness can also be easily adjusted by different curing temperatures. Finally, it should be noted that the bulk properties of a biomaterial usually do not change, because surface modification only changes the outermost surface composition.<sup>113</sup>

Another important note regarding chemical reaction of cells and tissue responses to both PAM and PDMS is that although PAM has adjustable stiffness values, it does not promote protein adsorption or cell adhesion due to its hydrophilic nature. PDMS has shown similar low cell adhesion and proliferation behavior like PAM, however, this time due to its high hydrophobicity.<sup>104</sup>

## Hazard assessment in terms of genotoxicity

A substance with the property of genotoxicity is called a genotoxin, *i.e.* a substance that has adverse effects on the integrity of the cell's genetic material (DNA, RNA). This may result in various somatic or germline mutations, or chromosomal aberrations all of which imply a substantial risk for developing abnormal phenotypes at the cellular and tissue level, and eventually at the organism level, *e.g.* apoptosis and cancer.<sup>114</sup>

Rapid progress in the development of new technologies in bioengineering and tissue engineering, especially to obtain scaffolding systems with properties similar to the ECM, is leading to comprehensive evaluations of genetic changes in growing cells in contact with an experimental material intended for use in clinical practice. Recent research has raised concerns about the use of biomaterials because of epigenetic changes. According to its definition, epigenetics means any heritable alteration in genetic expression without changes in

the DNA nucleotide sequences. The main alterations include DNA methylation and histone modifications (*e.g.* methylation and acetylation), and these can result in a broad range of biological processes and diseases. Collectively, these dynamic changes are very specific to tissues and stages of development in response to internal and external stimuli, such as toxicant materials.<sup>115,116</sup> These complicated patterns of epigenetic change highlight the importance of epigenomic profiling, with a particular focus on methylation, to answer biological questions.

Currently, most common epigenomic technologies are used to characterize nucleosome-free regions (DNaseI-Seq; MNase-Seq; FAIRE-Seq; ATAC-Seq), protein-mediated DNA interaction sites (Hi-C; 5-C), histone markers and DNA-binding proteins (ChIP-Seq), and DNA methylation (array hybridization, WGBS, MBD-Seq, PacBio, nanopore).<sup>117</sup> The use of high-throughput omics technologies to assess potential hazards of materials in tissue engineering is necessary to manage adverse effects on cardiac tissue engineering, especially in light of the scarce literature on this topic. To the best of our knowledge, genotoxicity tests of materials have been limited to traditional molecular techniques such as PCR, while in the era of omics, large-scale massively parallel sequencing, or next-generation sequencing (NGS), have been commercially available for around 10 years and have dramatically changed our understanding of mutagenesis.<sup>118</sup>

A major challenge in material toxicology is posed by the considerable discrepancies among toxicity studies, owing to different intrinsic properties of materials, cell culture media, and dispersion methods. The latter may have a substantial impact on research results.<sup>119</sup> Therefore, a critical step in hazard analysis is the comprehensive assessment of the frequency of somatic mutations in cells after treatment with potentially genotoxic agents or in biopsied tissues from individuals who may have been exposed to such agents. The mutational signatures of genotoxic agents tested in target cells or tissues will help to further elucidate their mechanisms of action.

## Sterilization of polyacrylamide and polydimethylsiloxane

Sterilization is a process to inactivate viable microorganisms such as fungi and bacteria, as well as spores and viruses.<sup>120</sup> Hydrogels in general, and biodegradable scaffolds specifically, have a high potential for contamination with a variety of living microorganisms, and sterility is a critical condition for the *in vitro* and *in vivo* use of scaffold materials. Scaffold sterilization must not alter or damage its biochemical, mechanical or structural properties; ideally sterilization should not affect any of these parameters.<sup>121</sup>

In addition to concerns regarding changes in physicochemical properties caused by different sterilization methods, remaining toxic residues and incomplete elimination of microorganisms are other important concerns in the search for optimal sterilization techniques.<sup>122</sup> It is evident that there is



no perfect method for sterilization. Therefore, the conditions of different methods should be carefully monitored, and advantages and disadvantages should be considered in light of the specific experimental requirements.

Below we provide an overview of the methods used to sterilize synthetic scaffolds, with emphasis on PDMS and PAM. The classical approach to sterilization is the use of heat, in two key ways: steam (125 °C to 134 °C for up to 20 min) and dry heating (160 °C for 120 min or 180 °C for 30 min). Although heat is able to remove all types of germs, care should always be taken regarding its side effects, such as alterations in mechanical strength and molecular weight.<sup>123</sup> Moreover, dry heat is not suitable for hydrogels or any wet material in general, and heat exposure will result in irreversible denaturation of the matrix proteins used for surface modification. The unmodified polymer itself withstands steam sterilization, as has been shown for PDMS.<sup>124</sup>

Features that have made irradiation approaches promising candidates for sterilizing biodegradable scaffolds are the low cost, short processing time, and low temperature. Irradiation methods are categorized as ionizing radiation techniques, which include gamma (dosage, 10–30 kGy) and electron beam (dosage, 25–150 kGy) irradiation, and UV (wavelength 200–280 nm), with exposure times ranging from minutes to hours.<sup>122,125</sup>

A more recent study evaluated the structural modification of PDMS with a molecular weight of 35 kDa by different doses of gamma irradiation. Elastomeric structures with different cross-linked density values were studied as a function of the gamma irradiation dose (250, 300, 350 and 400 kGy). A significant correlation was reported between thermomechanical behavior and the irradiation dose. Thermal stability showed an interesting behavior that indicated a direct correlation between the decomposition temperature and the structure generated by gamma irradiation.<sup>126</sup>

Gamma irradiation probably induces the formation of reactive species with long-term effects. In polymers, high doses of radiation can cause brittleness and cracks on the surface or promote cross-linking, and even ordinary doses affect the polymer molecular weight, molecular weight distribution, and the physical properties of the treated material.<sup>127,128</sup>

High energy radiation of commercial PDMS promotes the formation of an infusible and insoluble 3D gel network. Radiation doses beyond the critical gelation dose cause increase in the gel content resulting in the soluble fraction and rubber-like behavior.<sup>129</sup>

Small-angle neutron scattering and UV-visible spectroscopic techniques were used to study the effects of gamma irradiation on microstructural modifications in PAM hydrogels. This treatment resulted in the presence of nanometer and sub- $\mu\text{m}$  inhomogeneities, and the size of these inhomogeneities was reduced at lower doses.<sup>130</sup>

Microorganisms show different sensitivities to UV exposure depending on the exposure time and wavelength. The most used wavelength of UV is 260 nm. In the laboratory, UV irradiation is often used to eliminate microorganisms on surfaces. This approach is particularly useful to reduce germs

on 2D substrates such as films and foils. For porous hydrogels, UV irradiation is not sufficient and does not eliminate microorganisms inside the scaffold.<sup>121</sup> Furthermore, the physicochemical properties of the scaffold may be adversely affected. Because of these undesirable reactions, the optimal UV conditions must be carefully evaluated before full operation. In general, UV irradiation is not the method of choice to sterilize hydrogels.<sup>121</sup>

Plasma technology, a recent method of scaffold sterilization, involves the use of ions including photons, electrons, positive and negative ions, atoms, free radicals, and nonexcited molecules.<sup>131</sup> Low temperature, improved cell interactions, increased wettability of the surface of biodegradable polymers, and straightforward protocols are among the advantages of gas plasma, although drawbacks such as alterations in the chemical and mechanical properties of the polymer and the formation of reactive species should be considered. Bertoldi and colleagues found that the common combination of hydrogen peroxide vapor followed by low-temperature gas plasma is effective in sterilizing polyurethane foams, but reported some degree of material degradation.<sup>132</sup> Controlling the exposure time, power, and temperature is important to achieve optimal elimination of a broad range of microorganisms and spores with minimal side effects.<sup>133</sup> As plasma sterilization operates under vacuum conditions, it is only suitable for PDMS.

Recently, supercritical carbon dioxide ( $s\text{CO}_2$ ) has become popular for the sterilization of delicate materials. The  $s\text{CO}_2$  method has long been used in the laboratory to dry biological samples for electron microscopy, and its use to eliminate microorganisms from hydrogels is now being explored, with excellent results.<sup>134</sup> Supercritical carbon dioxide is able to penetrate deep into the material and reach the inner hydrogel structure. Jiménez *et al.* have shown that  $s\text{CO}_2$  efficiently eliminates bacteria while leaving the physicochemical properties of poly(acrylic acid-co-acrylamide) hydrogels almost entirely unaltered.<sup>135</sup> The efficiency of this method to reduce pathogens has been investigated in recent years, and  $s\text{CO}_2$  was shown to substantially reduce germs although it requires additives to reach its full potential.

One additive that can be used for sterilization is peracetic acid (PER). The oxidizing effects of PER increase the effectiveness of sterilization, but may oxidize proteins or peptides used to coat the scaffolds. Although PER bears the risk of oxidizing amino acids, it was found that epidermal growth factor can be sterilized by  $s\text{CO}_2$  with the addition of PER without losing its biological effect.<sup>136</sup> In conclusion,  $s\text{CO}_2$  with the addition of PER can be considered a powerful yet gentle method to sterilize hydrogels, even with peptide- or protein-based surface modifications.

## Cardiovascular applications of other biomaterials

Here, we present a brief overview of other biomaterials especially in terms of cardiovascular applications.



The two main classes of scaffolds used in cardiac tissue engineering are synthetic and natural materials. Natural materials readily provide the necessary signals to cells through interactions between intercellular receptors and reacting with matrix molecules. For example, hydrogels derived from natural materials are suitable to apply in tissue engineering applications because of their adjustable mechanical to the natural matrix of the heart.<sup>137</sup>

The most widely used natural materials are collagen, fibrin, hyaluronic acid (HA), preparations of matrix from Engelbreth-Holm-Swarm-tumor cells (Matrigel) and preparations of native heart matrix.<sup>138–143</sup>

Biomaterials based on collagen are widely used in cardiac tissue engineering due to its specific physical and chemical properties and rare immunogenicity. Collagen based matrix products are already FDA approved. One important point to consider is the relationship between this natural biomaterial and the target cells, which is of particular importance in iPSC-derived cell populations. Moreover, the purity and batch-to-batch variability are additional critical factors that should be considered.<sup>144</sup> Of note, cardiomyocytes do not interact directly with collagen type I and connective tissue cells are required as adaptors.<sup>145</sup>

Fibrin-based cardiac tissue engineering applications can be a way to solve problems related to cell survival, distribution, delivery of growth factors and revascularization. In addition, the possibility of obtaining it from an autologous source or the possibility of creating a delicate and adjustable structure by changing the conditions of its polymerization are advantageous. It should be noted that new technologies can be used to change its geometric coordinates.<sup>146</sup>

Hyaluronic acid, which is synthesized by hyaluronan synthases and in normal tissues has different significant roles such as angiogenesis, homeostasis and altered viscoelasticity of extracellular matrix. HA physicochemical properties such as solubility and the availability of reactive functional groups have facilitated its chemical modifications, which has made it an important biocompatible biomaterial for tissue engineering applications. It should also be noted that hyaluronic acid-based biomaterials as well as related bioscaffolds do not cause any allergic reactions or inflammation.<sup>147</sup> Yoon *et al.* reported regenerative effects by using hyaluronic acid-based injectable hydrogels in an ischemic heart model.<sup>148</sup>

It has been revealed that neonatal rat cardiomyocytes loaded in Matrigel and applied *in vivo* can be vascularized and mature indicating that immature cells that have received appropriate cues in this scaffold can form tissue with myocardial features.<sup>149</sup>

Furthermore, the most commonly used synthetic materials are polyesters such as poly(lactic acid), poly(glycolic acid) polylactones, and polyurethanes.<sup>150–153</sup> These materials are readily achievable but may be restricted in cellular reactions and therefore often altered by binding to adhesive peptides or releasing biomolecules.

Poly(lactic acid) (PLA) is a synthetic biomaterial-synthesized either by ring opening polymerization or polycondensation – has

been shown has a wide range of applications in tissue engineering. It is convenient for medical applications because of degradation into lactic acid which is a metabolite product.<sup>154</sup> Many studies have been performed on the use of poly(lactic acid) (PLA) in cardiac tissue engineering. Although there have been barriers such as hydrophobicity and functionalization problems but by combining it with other biomaterials such as poly(glycerol sebacate) these barriers are partially resolved.<sup>155</sup>

As a biodegradable and biocompatible polyester polyglycolic acid has been approved by the FDA for use in biodegradable structures. Although its use has always been considered in cardiac tissue engineering, the hydrophobicity of its surface has limited cell attachment and cell migration. Further studies are needed to investigate the physicochemical properties and behaviors of derived scaffold composites.<sup>156,157</sup>

Recent advances in synthetic chemistry have played an important role in the production of hybrid biomaterials with extraordinary conductivity and strength.<sup>158</sup> For example, in a study by Shevach *et al.* gold nanoparticle-decellularized matrix hybrids were developed in terms of cardiac tissue engineering. They showed that cardiac cells engineered within the hybrid scaffolds have elongated and aligned morphology, massive striation, and organized connexin 43 electrical coupling proteins. Moreover, the hybrid patches revealed more suitable function in comparison with pristine patches, such as more powerful contraction force, less excitation threshold, and faster calcium transients.<sup>159</sup>

In another example carbodiimide-based sequential crosslinking technique was applied to produce aortic valve extracellular matrix (ECM) hybrid scaffolds from collagen type I and HA. The resulting hybrid showed an extensive range of pore size (66–126  $\mu\text{m}$ ) which is suitable for valvular tissue regeneration.<sup>160</sup>

Polyester urethane (PU) also is biocompatible, biodegradable, and flexible with excellent mechanical properties which have introduced it as a major class of elastomers in tissue engineering. The stiffness of the heart muscle at the beginning of diastole varies from 10 kPa to 500 kPa at the end of diastole so an elastomer such as PU that can provide a stiffness in this range may be beneficial in cardiac tissue engineering.<sup>161</sup>

Insufficient conductivity can be a serious drawback of both synthetic and specially natural biomaterials in the cardiac tissue engineering but recently the use of nanostructured polymers and polymer nanocomposites caused a revolution in the cardiac tissue engineering field and improved electrical and mechanical properties of biomaterial resulting in promoted tissue growth as well.<sup>153</sup>

There are many studies on the production of conductive hydrogels, which are obtained by combining soft hydrogels and conductive polymers. The controlled electrical properties are useful for the total process of tissue formation.<sup>154</sup> For example, adding carbon nanotubes into PDMS can enhance both thermal and electrical properties.<sup>155</sup>

Furthermore, Hosseinzadeh *et al.* engineered poly acrylic acid-based hydrogels to create nanofibers using aniline polymerization approach. The resulted composite had a stable electrical conductivity for biological applications.<sup>162</sup>





## Conclusion

The complex relationship between cell networks and hydrogels can potentially influence cardiac-based tissue engineering systems. Hence, a precise understanding is needed of cardiac tissue, especially the components in heterogeneous ECM, as well as detailed knowledge of the structural and chemical properties of biomaterials and how they may react to different sterilization methods. This information can provide ways to control mechanical, compositional, and structural cues in ways the most closely represent the features of the main tissues with minimal hazards. The research reviewed here shows that PDMS and PAM are versatile biomaterials especially suitable for the *in vitro* study of cardiomyocyte physiology.

## Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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