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Guillaume Conzatti, Sandrine Cavalier, Christèle Combes, J. Torrisani, Nicolas Carrère, Audrey Tourrette

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Guillaume Conzatti, Sandrine Cavalier, Christèle Combes, J. Torrisani, Nicolas Carrère, et al.. PNIPAM grafted surfaces through ATRP and RAFT polymerization: Chemistry and bioadhesion. Colloids and Surfaces B: Biointerfaces, 2017, 151, pp.143-155. 10.1016/j.colsurfb.2016.12.007 . hal-01625967

HAL Id: hal-01625967

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To link to this article : DOI: 10.1016/j.colsurfb.2016.12.007
URL : http://dx.doi.org/10.1016/j.colsurfb.2016.12.007

To cite this version : Conzatti, Guillaume and Cavalier, Sandrine and Combes, Christèle and Torrisani, J and Carrerre, N and Tourrette, Audrey *PNIPAM grafted surfaces through ATRP and RAFT polymerization: Chemistry and bioadhesion*. (2017) Colloids and Surfaces B Biointerfaces, vol. 151. pp. 143-155. ISSN 0927-7765

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PNIPAM grafted surfaces through ATRP and RAFT polymerization: Chemistry and bioadhesion

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Keywords:
Surface functionalization
Reversible addition fragmentation chain transfer polymerization
Atom transfer radical polymerization
Poly(*N*-isopropylacrylamide)
Bioadhesion

A B S T R A C T

Biomaterials surface design is critical for the control of materials and biological system interactions. Being regulated by a layer of molecular dimensions, bioadhesion could be effectively tailored by polymer surface grafting. Basically, this surface modification can be controlled by radical polymerization, which is a useful tool for this purpose. The aim of this review is to provide a comprehensive overview of the role of surface characteristics on bioadhesion properties. We place a particular focus on biomaterials functionalized with a brush surface, on presentation of grafting techniques for "grafting to" and "grafting from" strategies and on brush characterization methods. Since atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization are the most frequently used grafting techniques, their main characteristics will be explained. Through the example of poly(*N*-isopropylacrylamide) (PNIPAM) which is a widely used polymer allowing tuneable cell adhesion, smart surfaces involving PNIPAM will be presented with their main modern applications.

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1. Introduction

Current developments in medicine have led to a need for new biomaterials in applications involving innovative strategies for disease treatment. These biomaterials have to fulfil various requirements depending on the application but in all cases biocompatibility is a crucial point that has to be considered in early processing stages. Biocompatibility is defined as the ability to act in a living system without any toxicity or rejection, whether physiological or immunological. Although biocompatibility includes "non-toxicity", these two concepts still remain differentiated. Sur-

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face functionalization of a material is sometimes necessary to avoid strong inflammatory responses and improve biocompatibility.

On the other hand, surfaces not only act as passive interfaces between the body (immune system, blood, cells) and the biomaterial but also take an active part in cell spreading, proliferation, differentiation and migration: all of these phenomena are intensively linked to surface/cell interactions. Thus, surfaces play a crucial role in the function of the biomaterial and can be used, for example, as activators of cells for tissue reconstruction (tissue engineering) [1]. In this field, cell adhesion to the surface is a key factor that must be carefully considered. Bioadhesion is defined as the adhesion between a biological entity, e.g. cells or tissues, and a surface. It is a complex phenomenon that involves many parameters. For a long time, it was difficult for scientists to clearly identify the mechanisms underlying cell adhesion, but they are becoming increasingly well understood. The interest of scientists for bioadhesion is not only theoretical but is also essential for the wide spectrum of applications depending on cell/surface affinity.

In cell sheet engineering, surface properties are used to control cell adhesion, so the living sheets can be stripped off easily. It was shown that cell sheets integrate well into tissues [2] and are promising tools for tissue reconstruction. Smart surfaces tunable between "on" (adhesive) and "off" (non-adhesive) states are then feasible. Naturally, the study of thermosensitive materials has been widely reported in the literature. Poly(N-isopropylacrylamide) (PNIPAM) is a promising polymer as a change of its hydrophilic interactions, and indirectly bioadhesivness, takes place between the room temperature and body temperature. More precisely, its lower critical solution temperature (LCST) occurs at 32 °C. While the monomer is cytotoxic, the polymer shows no toxicity for the various cell types [3] and constitutes a good example to help understand bioadhesion mechanisms. Its characteristics make this polymer one of the most intensively studied in the literature whether grafted or coated onto material surfaces.

Plasma treatment is probably the most common process for surface modification, both for the introduction of functional groups or for coating the surface with polymer [4]. In the presence of air, oxygenated surfaces will be produced under the plasma, leading to a change in hydrophilicity [5]. This modification can involve an increase in cell adhesion, as is the case for polystyrene (PS) [6]. In addition, the plasma can etch the surface, enhancing its roughness. This modification of topography, as observed in plasma-treated poly(methyl methacrylate) (PMMA), tends to increase the cell affinity to these surfaces [7]. The introduction of reactive groups also permits compounds or chemical functions to be grafted onto the material surfaces. In the presence of oxygen, surfaces activated *via* the introduction of hydroxyl groups, can be grafted with monomers or polymers, such as NIPAM/PNIPAM though *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC)/N-hydroxysuccinimide (NHS) amide coupling [8]. Under argon plasma, radicals will be produced. These radicals can be used directly or, after exposition to air, create peroxides that can initiate polymerization [9].

While plasma treatment presents the advantage of being easy to perform on materials, only a low level of surface structuring is obtained. In addition, it was shown that plasma-deposited PNIPAM shows greater cell adhesion than e-beam coated PNIPAM [10]. These results emphasized the importance of the method used for surface modification. Control of the surface structure also seems to be a critical point. A brush structure, *i.e.* a self-assembled close-packed monolayer of polymer chains, provides precise control of the surface morphological properties. More specifically, it becomes possible to control the thickness and the density of the grafted layer. As a result, these structures are now extensively used, especially for biomedical applications, and display good performance in terms of bioadhesion [11].

To obtain controlled brushes, a well-defined grafted polymer is necessary. Controlled radical polymerization can be used to control polymer grafting using various techniques. The most popular ones are ring-opening polymerization, nitroxide-mediated polymerization, reversible addition fragmentation chain transfer (RAFT) and atom transfer radical polymerization (ATRP). A review of functional polymer brushes produced by controlled radical polymerization was published by Olivier *et al.* [12]. RAFT polymerization and ATRP are widely used as they are versatile and reduce poly-dispersities. They rely on the equilibrium between dormant and active species, so are sometimes called "living" polymerizations.

It is necessary to understand cell adhesion mechanisms to design a biomaterial with tunable bioadhesion properties. The aim of this review is not to be exhaustive on surface modification processing but to give a comprehensive multidisciplinary overview of the cell/surface adhesion mechanisms as well as the chemical engineering of surfaces. Hence, an introduction to cellular biology and bioadhesion will be made. A second part will deal with brushes and the chemical routes used to obtain such structures. More precisely, ATRP and RAFT polymerization methods will be reviewed along with the ways in which they have been characterized. Finally, engineering of tunable surfaces using PNIPAM will be presented with its main and most recent applications.

2. Bioadhesion

2.1. Bioadhesion mechanisms

With a size from 1 to 100 μm, cells are composed of various entities. Some are dedicated to its structure: a phospholipid bilayer, the cell membrane, maintains the separation between the cytosol (internal liquid phase) and the extra-cellular matrix (ECM) and the cytoskeleton, composed mainly of microtubules and actin filament networks, control the rigidity of the structure [13]. The ECM composition varies depending on the tissue concerned. It contains a number of proteins: fibronectin, collagen, laminin, but also growth factors and all the proteins needed for cell support and inter-cell communication. Actin filaments assemble to constitute the cytoskeleton. They are connected to integrins (transmembrane glycoproteins) through vinculin and talin (Fig. 1) [14,15]. These integrins specifically bind to ECM proteins such as fibronectin (or vitronectin) through arginylglycylaspartic acid (contraction of L-arginine, glycine, and L-aspartic acid, abbreviated RGD) coupling. These integrin/ECM protein interactions are responsible for cell adhesion to surfaces (intercellular cohesion is regulated by other mechanisms, *e.g.* cadherin-mediated homotypic junctions). This adhesion is commonly divided into the different phases described in Fig. 2a [16]. The first seconds of contact are characterized by the formation of non-specific interactions. Then, biological interactions occur, including adhesion protein/fibronectin interactions. This second step leads to a cascade of actions including the reorganization of the cytoskeleton and clustering of integrin receptors. Consequently, cells contract their cytoskeleton to maintain a mechanical state of tension, also called prestress. Later, cells produce ECM to reinforce their integration and maintain a propitious environment. It is thus obvious that the adsorption of adhesive proteins is a key point for cell adhesion.

2.2. Surfaces and bioadhesion

Two main parameters will determine the behavior of implanted biomaterials: (1) their bulk properties, especially the rigidity, plays a role in the quality of the implantation into tissues, and (2) their surface properties control the immune system response (called immunogenicity), the destruction of cell integrity, and the bioad-

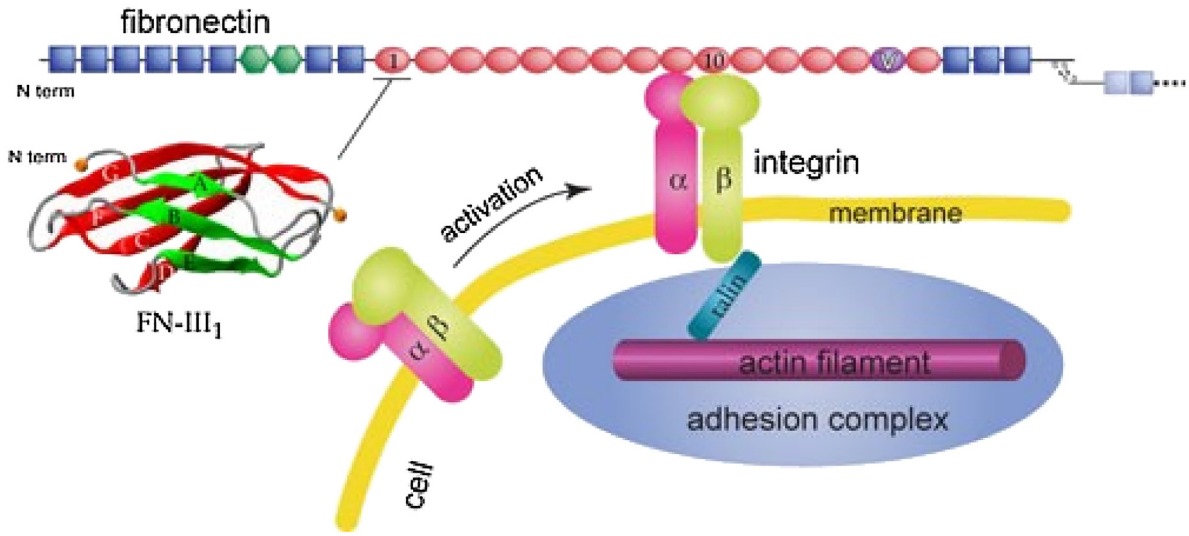

Fig. 1. The adhesion structure of a cell in a matrix. The matrix is linked to the cytoskeleton through integrins and talins. Reproduced from Ref. [14] with the permission of The Royal Society of Chemistry.

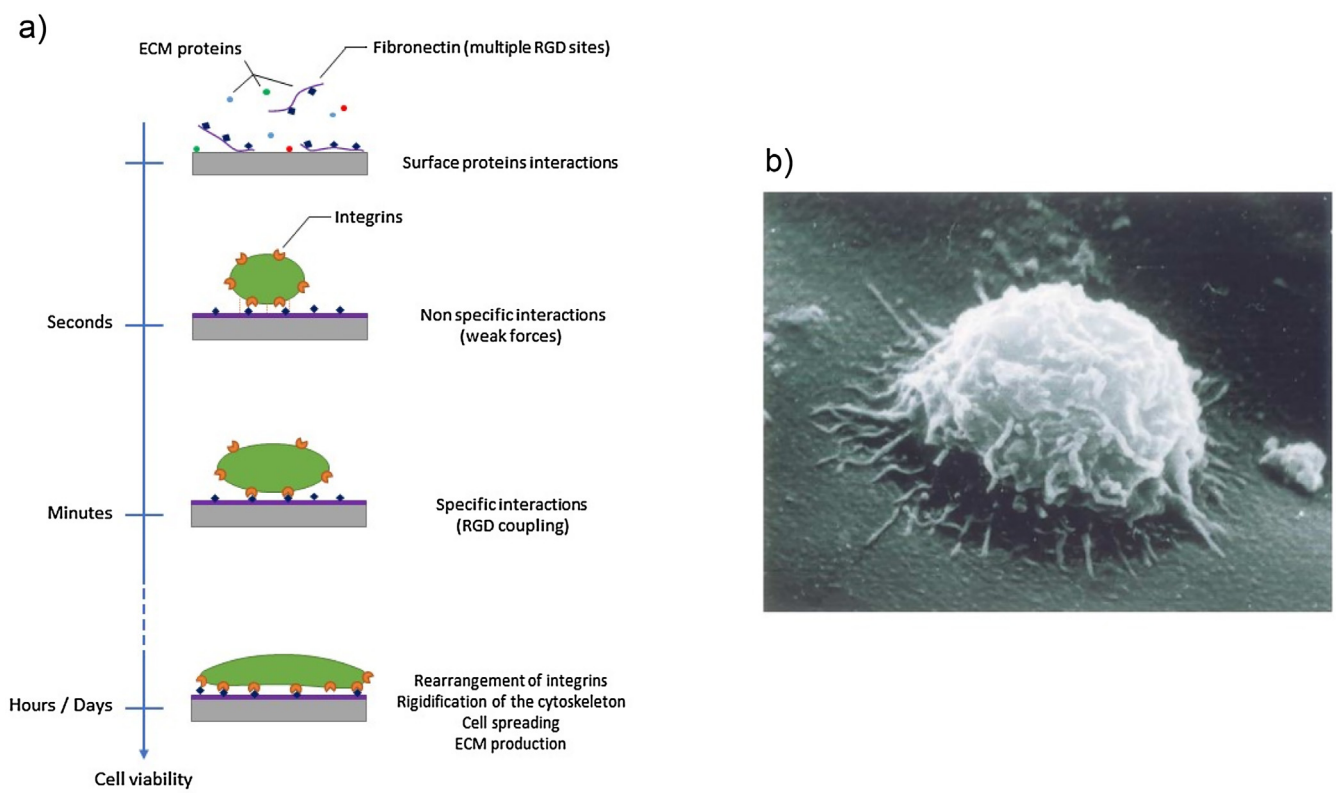

Fig. 2. (a) Stages of cell adhesion: interactions and kinetics. (b) SEM picture of a myoblast cell on an artificial surface, 2001. Fig. 2b is reprinted from Ref. [17], Copyright (2002), with the permission of Elsevier.

hesion. As cell membranes are anionic, cationic surfaces have to be carefully used since they can damage cells and tissues, whereas negatively charged polymers will electrostatically repel cells [18–21]. The importance of surface/cell interactions has been investigated for many years, and an interesting discussion was published in 2001 by Castner *et al.* [17]. In this aim, synthetic materials and bioadhesion have been studied and observed by scanning electron microscopy (SEM, Fig. 2b).

An important point is to realize that in a living system surfaces are spontaneously covered by proteins. It is now better understood that cell adhesion is both ruled by (i) specific (biological) interactions (receptor/ligand) and (ii) the physico-chemical properties of the material. Hydrophilicity, mechanical properties and morphology are among the parameters involved in the bioadhesion process.

As mentioned above cell adhesion between tissues and ECM is possible through the specific binding of integrin with fibronectin (Fig. 1) [22]. In 2011, Pei *et al.* observed the importance of specific interactions (*i.e.* fibrinogen RGD coupling) in the process of attachment between cells and their substrate [23]. They noted the number of human foreskin fibroblast (hFF) cells and how they

spread on poly(ethylene glycol) (PEG) brush surfaces (see Section 3.1) grafted onto TiO_2 and compared pre-treated hFF (blocked integrin bonding-sites) and non-treated hFF (free integrin bonding sites).

The number of attached cells strongly decreased when the integrin bonding sites were blocked. Nevertheless, weak non-specific interactions occurred when there was a high level of affinity between the cells and the biomaterial. This study also provides precious information on the influence of the PEG brush density. PEG is an anti-fouling polymer. At high grafting density, PEG chains are collapsed in a "brush regime" ($L < 2Rg$, with L the distance between two neighboring chains and Rg the radius of gyration of polymer chains), whereas a "mushroom regime" is observed at lower values ($L > 2Rg$). A gradient of PEG was used to observe its effect on protein adsorption and cell adhesion. For short experiments, as the density of PEG increased, a strong decrease of the number of adhered cells occurred when the brush regime was reached and a correlation established with the protein adsorption profile. Fibroblast saturation occurs in the "brush regime", meaning that even a low amount of fibrinogen (2.2 ± 3.4 ng/cm2) is sufficient to activate adhesion. Here the importance of protein/cell specific interactions is obviously crucial.

A model involving a protein layer between biomaterials and cells was established in the early 2000's [1,17,24]. According to this model, cell/matrix adhesion depends on the ability of the surface to adsorb proteins without modifying their native structure. Denaturation is brought about by the water structure near the surface, *i.e.* the hydrophilicity of the superficial layer on the material. A review, published in 2011 dealt with the concept of native immune system response (cascade system) [25]. The article emphasized the importance of the non-denaturation of protein structure at the material/body interface to avoid activation of the primary immune system. In 1998 Volger *et al.* already underlined the importance of surface chemistry in terms of hydrophobic/hydrophilic properties [26]. This group showed that water/surface interactions are of interest. As criteria of hydrophilicity/hydrophobicity they used contact angle measurements. A strongly bound water to a surface (*i.e.* hydrophilic surface) cannot be removed. This will avoid interactions directly between a biological entity (*e.g.* a protein), and the surface, leading to low or absence of adsorption [27]. Moreover, it is widely accepted that moderately hydrophilic materials are suitable for cell adhesion, with a contact angle around 70–80° [28,29]. For example, endothelial cells show good attachment to polycaprolactone-grafted-poly(methyl methacrylate) (PCL-g-PMMA) surface with a contact angle around 70°. Additionally, it is now accepted that surfaces which are too hydrophobic lead to the denaturation of proteins [1,28]. More precisely, hydrophobic and hydrophilic amino acids, constitutive of proteins, rearrange their organization depending on the surrounding media and thus hydrophobic surfaces can favor the externalisation of hydrophobic moieties, leading to unfolded proteins [30]. Regarding this assessment, post-treatments (*e.g.* plasma treatments) are sometimes used to increase surface hydrophilicity through the introduction of polar functional groups. In contrast, it is known that the interactions of highly hydrophilic surfaces with ECM adhesion proteins are weak [6]. Keselowsky *et al.* characterized various functional groups on the criteria of fibronectin adsorption (in increasing order): $OH < COOH < CH_3 < NH_2$. On the other hand, adhesion of osteoblasts increases as follows: $CH_3 < NH_2 = COOH < OH$ [31]. This trend inversion can be explained by the geometrical deformation of ECM proteins, *i.e.* denaturation. On brush surfaces, the optimal contact angle depends not only on the nature of the matrix but also on the method of surface modification. This can be due to the influence of chain length and density of the grafted polymer on the conformation of the protein adsorbed on the surface. For instance, it has been pointed out that when Fe^{2+} is used as initiator for the graft polymerization of PMMA on poly(L-lactic acid) (PLLA) surfaces, maximum chondrocyte attachment is obtained when the contact angle is 52°, whereas UV-initiated surfaces are optimal for a contact angle of 76° [28]. The authors suggest that the difference of biological properties between the two PLLA grafted PMMA could be due not only to surface wettability (contact angle) but also to the higher density, uniformity and shorter chains of iron-initiated polymerized surfaces. The wettability criterion is obviously strongly limited as the surface mechanical properties and structure, cell types and charge density are ignored. For example, Bacakova *et al.* showed that soft matrices are not favorable for cell adhesion [6]. They explained that ECM deposited on such surfaces is not able to resist the forces involved during cell focal point formation.

Indeed, specific interactions are not enough to described adhesive phenomena entirely and physico-chemical properties have to be added to the equation. Hence, the internal organization of cells is remodelled throughout their life and is strongly influenced by the surrounding medium not only *via* chemical stimulation but also by mechanosensing, until a morphological equilibrium is reached [13,32]. Thereby, the shape of cells, as well as their rigidity and motility depend on their support. For example, many cells have the ability to sense the stiffness, by applying a stress, of their external environment. These cells include brain, muscle, neurons and many other cell types [33]. Cells probe the surface through myosin and actin filament cross-bridging and a stiffness control loop is set up: cytoskeleton and adhesion will adapt depending on the feedback. As a consequence, stiffer matrices lead to an increase in the elasticity of the cells, and can be measured by atomic force microscopy (AFM) [34] or, more gently, by indentation with optical tweezers [35]. However, rigidity scanning of the cell substrate is a time-consuming process, taking from minutes to hours [13], thus viscosity can be considered, in the case of a gel for example. Sometimes, cross-linking can improve cell activity on a gel [36]. In addition, mechanosensing can be an initiator of cell displacement on surfaces (mechanotaxis, discovered by Lo *et al.* [37]), from the soft to the stiff [38] and motility were shown to be linked to focal contacts and thus indirectly to cellular adhesion [39,40].

Moreover, depending on their type, cells will not behave in the same way depending on the elasticity of the biomaterial. For instance, on soft matrices, fibroblasts adhere in a labile way whereas on stiffer materials they make stable focal points (adhesion) and rigidify their cytoskeletons. Consequently, the motility of fibroblasts on stiff materials is reduced [38]. The mobility of the chains that constitute the brushes could also lead to superficial mechanical instability and thus to lower adhesion [41].

Despite the adhesion of cells, the biomaterial can also disturb cell activity. A perturbation in the exocytosis response of cells is revelatory of this perturbation in ways that can, for instance, be measured by histological studies or carbon-fibre microelectrode amperometry (CFMA), as observed by Reed *et al.* [10]. In this study, the introduction of PNIPAM (through plasma deposition or spin coating) on surfaces have shown to slow down the cell exchange between vesicles and extracellular space. Additionally, spin-coated PNIPAM were shown to hyper-activate the exocytosis activity of cells, whereas plasma deposited PNIPAM only affected kinetics. If this hyper-activation by the surface can present harmful effects, some studies seek for accelerating tissue regeneration by modifying the surface, such as observed by the introduction of free amino group on PLLA surfaces [42]. The surface architecture is also a key point. 3D architectures are commonly modulated in the field of tissue engineering, as they reproduce a more realistic natural biological environment. A nice review of this topic was published by Abbott and Kaplan in 2015 [43].

In conclusion, the surface hydrophilicity (presence of functional groups), the surface structure and stiffness are characteristics that must be considered for efficient bioadhesion. However, in the

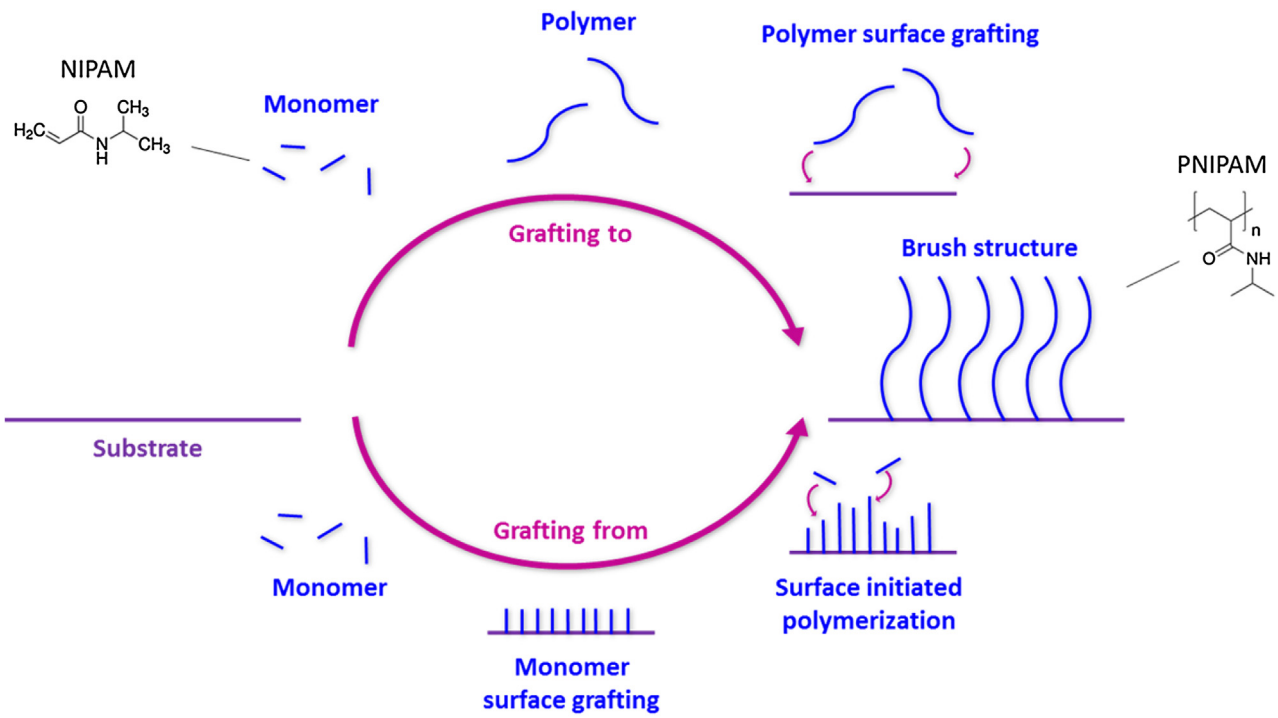

Fig. 3. Two different strategies of polymer grafting (example of PNIPAM): the surface initiated polymerization of a monomer ("grafting from") or the grafting of the polymer ("grafting to") on a surface to obtain a brush structure.

present review, we only consider surface modification that does not involve any bulk modification, in particular in term of biomaterial stiffness, and the effect of brush structures on mechanical surface properties will not be discussed. Below, we focus on a key step, *i.e.* the choice of surface modification method used to obtain brush structures on material surfaces that enable good control of the surface state and properties.

3. Surface modification

3.1. Brush structure

As discussed above, the control of biomaterial surfaces is a challenge for scientists involved in the development of medical devices. Brush structures in particular are interesting in terms of protein penetration and calibration studies [44]. Thanks to recent developments in chemistry, various techniques are available to graft or coat biomaterials with polymers. For biological applications, covalent grafting seems to be the best choice compared to physically grafted systems, due to risks of desorption with this latter method. Polymer brushes, which consist on a thin film of self-assembled polymers, are of interest [10,45]. Wettability, but also the variety of possible end-group functions, the substrate and the chemistry are the main points that make these systems attractive. The modularity of the polymer brush synthesis is illustrated by the broad range of systems that have been developed in recent years: uniformed, patterned, or gradient (in terms of density or chemical composition) brush layers have been prepared with one or several polymers [12,45].

Two different approaches can be considered, namely "grafting to" (*i.e.* to the surface) and "grafting from", as shown in Fig. 3 [45,46].

The "grafting to" method consists in coupling an end-functionalized polymer and a reactive surface. This approach yields well-defined grafted polymer. However, the deposited layers have low densities and their thickness is limited (100 nm [47]) due to the steric hindrance during grafting and diffusion processes [48–51].

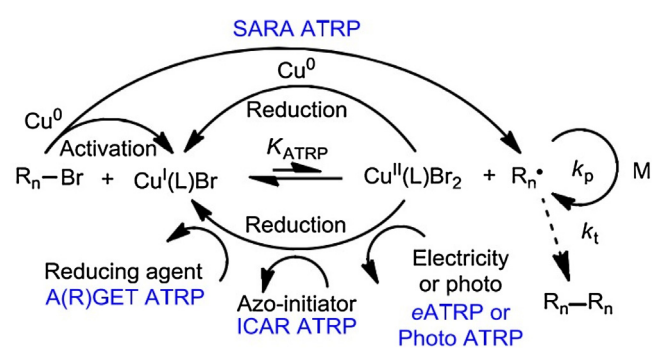

Fig. 4. Advanced ATRP mechanisms catalyzed by Cu(I)/Cu(II) complexes. Reprinted from Ref. [54], Copyright(2014), with permission of Elsevier.

The "grafting from" method consists in polymerization directly from the surface. Several techniques are based on this process. Surface-initiated polymerization (SIP) based on radical chemistry is commonly used [47,51,52]. "Grafting from" techniques yield higher grafted layer densities and overcome thickness limitations. These advantages made this strategy the most widely adopted. However, the characterization of grafted chains is more difficult and, apart from model surfaces, still remains a great challenge.

Various techniques are commonly used for grafting polymers to surfaces, both for "grafting to" or "grafting from" strategies [47,53,54]. Among these techniques, the most used, *i.e.* ATRP and RAFT, will be developed in the next sections.

3.2. Grafting techniques

3.2.1. Atom transfer radical polymerization (ATRP)

As is generally the case in controlled radical polymerization, ATRP chemistry relies on the equilibrium between active and dormant chains [46,47,55,56]. The mechanism is illustrated in Fig. 4. Surface initiated ATRP (SI-ATRP) is a "grafting from" technique that

consists in immobilizing a halogenated initiator on the surface, followed by ATRP. The main advantages of the ATRP are the close control of film thickness and chain length with low polydispersity [47]. It is also possible to control thickness and graft density separately, by modulating the reaction time and stoichiometric ratio, respectively [57,58]. In addition, ATRP is known to be versatile and easy to perform (mild conditions) [51,58,59]. While high temperatures make the reaction time shorter, some studies also show a slight reduction of the polydispersity [60]. Nevertheless, the need of metal catalysts is a limitation for biomedical applications. Basically, the metal is oxidized/reduced, and thus generates or absorbs a radical, leading to the activation/deactivation of polymer chains, respectively as shown in Fig. 4. At the present time, due to its high catalytic activity copper is used most [61,62]. Iron catalyzed-ATRP can be performed using low amounts of catalyst, reducing the toxicological risks as iron is considered less toxic and more environmentally friendly than copper [63–65]. Iron is also the most abundant metal on earth making it relatively cheap; these characteristics have initiated a lot of research and interest in Fe catalyzed organic chemistry, including ATRP, in line with the perspectives of "green chemistry" [66,67]. The choice of the ligand is a complex question; it depends on the nature of the polymer and the catalyst used [68]: for example, pentamethyldiethylenetriamine (PMDETA) can be used in combination with Cu [69], and tris(3,6-dioxaheptyl) amine (TDA) with Fe [70]. Furthermore, the activators regenerated by electron transfer (ARGET) ATRP, developed by Matyjaszewski *et al.* diminish the amount of catalyst needed (< 50 ppm) [71,72]. This advanced ATRP, derived from the AGET-ATRP (for activators generated by electron transfer ATRP) involving a reducing agent to initiate the reaction, consists in using an excess of reducing agent (*e.g.* environmentally friendly ascorbic acid [73]). This initiator does not only generate but also maintains a sufficient amount of Cu(I) (in the case of copper catalyzed ATRP) without the use of a radical organic compound which could lead to side reactions, cross-linking or the formation of new chains [55]. Another advantage of ARGET-ATRP is that the oxidized catalyst, *e.g.* Cu(II) or Fe(III), can be used directly without the need for early-stage reduction and careful handling. Finally, this technique increases the air tolerance and can avoid the need of a controlled atmosphere [74,75]. Ascorbic acid (also called vitamin C) is preferable to classic Sn reducing agents due to its non-toxicity towards human beings and the environment. However, ascorbic acid presents the disadvantage of being a strong reducing agent, so its use in water can lead to an extensive conversion of Cu(II) to Cu(I) and can diminish the control of ATRP. One solution would be to use a less efficient solvent, such as anisole, to decrease the reducing activity of the ascorbic acid [73,75].

Initiators for continuous activator regeneration (ICAR) ATRP use the addition of a free radical initiator such as azobisisobutyronitrile (AIBN) to (re)generate the active metal [63,71].

In supplemental activator and reducing agent (SARA) ATRP the reducing agent is M(0), *e.g.* Cu(0) for a copper catalyzed ATRP [67,76]. Using iron powder, the polymerization can be catalyzed by Fe(0) [60], with or without the use of Fe(III) salts but in this latter case a less controlled polymerization over time can occur [63].

Many ATRP elaborations are involved for surface modification, nonetheless we would like to mention biomedical uses of this chemical route: biofouling surfaces or membranes [77], double responsive cellulose membranes [78,79], cell attachment/detachment (through PNIPAM grafting) [69,80]. To perform a "grafting from", the idea is to chemically graft the initiator on the surface so the growth will be directly initiated on the material. This step is facilitated by the fact that ATRP initiators are acyl bromides. A self-assembled monolayer (SAM) is generally also grafted before the initiator. ATRP can be achieved in combination with plasma treatments to help initiator immobilization [81]. A few examples of SI-ATRP are given in Table 1.

ATRP commonly exhibits a pseudo-first-order kinetics, at least below high rates of conversion. The direct characterization of grafted polymer through the "grafting from" method is difficult. Generally, a sacrificial initiator is used, assuming that the polymer growth is similar on the surface and in the medium [51,52,58,59,82–85]. Another method is to use reversible or breakable surface bonds in order to detach and study the grafted polymer [86]. A more detailed discussion on brush characterization is given in Section 3.3.

3.2.2. Reversible addition-fragmentation chain transfer (RAFT) radical polymerization

Similarly to ATRP, RAFT polymerization is a living polymerization based on the equilibrium between active (*i.e.* bearing radicals) and dormant chains, and also shows pseudo-first-order kinetics. Initiation is performed in traditional ways, *e.g.* using thermal initiators such as azobisisobutyronitrile (AIBN) or 4,4′-azobis(4-cyanovaleric acid), which has the advantage of being carboxylic acid end-functionalized. A chain transfer agent (CTA) (also called RAFT agent) ensures this equilibrium during the propagation steps, as shown in Fig. 5 [87,88]. The reduction of active chain concentration results in a narrow distribution of the chain length, with a polydispersity index (PDI), for PNIPAM, able to reach values around 1.20, but PDI below 1.10 can be obtained in optimal conditions [89–91]. Additionally, RAFT polymerization can be achieved with a broad range of temperatures, from room temperature to 140 °C [92]. A higher temperature allows a shorter reaction time; lower polydispersities can sometimes be expected.

One of the main advantages of RAFT polymerization compared to ATRP is that it is a metal-free chemical route. In contrast, it can require the synthesis of the RAFT agent. In modern RAFT polymerization, this agent classically contains a thiocarbonylthio moiety, as for the commonly used trithio-carbonate and dithio-carboxylate type. As the RAFT polymerization process relies on the kinetics of addition and fragmentation of this agent, the choice of its substitute, classically called Z and R, is crucial. Z is dedicated to the activation of the double bond by stabilizing the adduct radical, R is a leaving group. A complete discussion about the choice of the RAFT agent is available in the literature [92]. The surface initiated RAFT (SI-RAFT) polymerization of PNIPAM can allow to obtain polydispersities below 1.3 [93]. Thanks to the sulfuryl groups of the CTA, elemental analysis can, in some cases, be used to determine the quantity of grafted RAFT agent. The theoretical molecular mass of the polymer, $M_{n_{th}}$, can be estimated through the following equation:

$$M_{n_{th}} = \frac{[Mono]_0}{[CTA]_0} \times M_{Mono} \times conv. + M_{CTA}$$

where M_{Mono} corresponds to the molecular weight of the monomer, $[Mono]_0$ to its initial concentration, *conv.* is the conversion rate of the monomer, M_{CTA} is the molecular weight of the CTA and $[CTA]_0$ its initial concentration [91,93].

In order to perform SI-RAFT polymerization, the initiator [90] or the CTA [93] have to be previously grafted to the surface. In the first case the homolytic cleavage of the initiator will lead to growth either on the surface or in the medium, both with the free CTA. In the case of CTA grafted surfaces, the initiator and another amount of the RAFT agent are introduced to permit the polymerization of free chains and their characterization. Few examples of SI-RAFT polymerization are given Table 1.

3.3. Characterization of brushes

Characterization of the "grafted from" polymer brush is a challenging task. In specific cases, the grafted chains can be removed from their substrate [86]. In other cases, free chains are generally

Table 1
Examples of surface functionalization with PNIPAM brushes using ATRP or RAFT polymerization process.

Radical polymerization	*Technic*	*Substrate*	*Solvent*	*Refs.*
ATRP	grafting from	polyethylene terephthalate (PET)	water	[94]
		Au	water/methanol	[95]
		graphene	water	[96]
		poly(ε-caprolactone) (PCL)	water/methanol	[69]
		parylene C	DMF/water	[80]
		Si	water/methanol	[97]
		cellulose	various solvents	[98,99]
		mesoporous Si films	water	[100]
RAFT polymerization	grafting to	Au nanoparticles	water	[89]
	grafting from	mesoporous Si nanoparticle	DMF	[101]
		aminated polyHIPE (high internal phase emulsions)	DMF	[93]
		cellulose	various solvents	[98,102]
		glass	dioxane	[90]

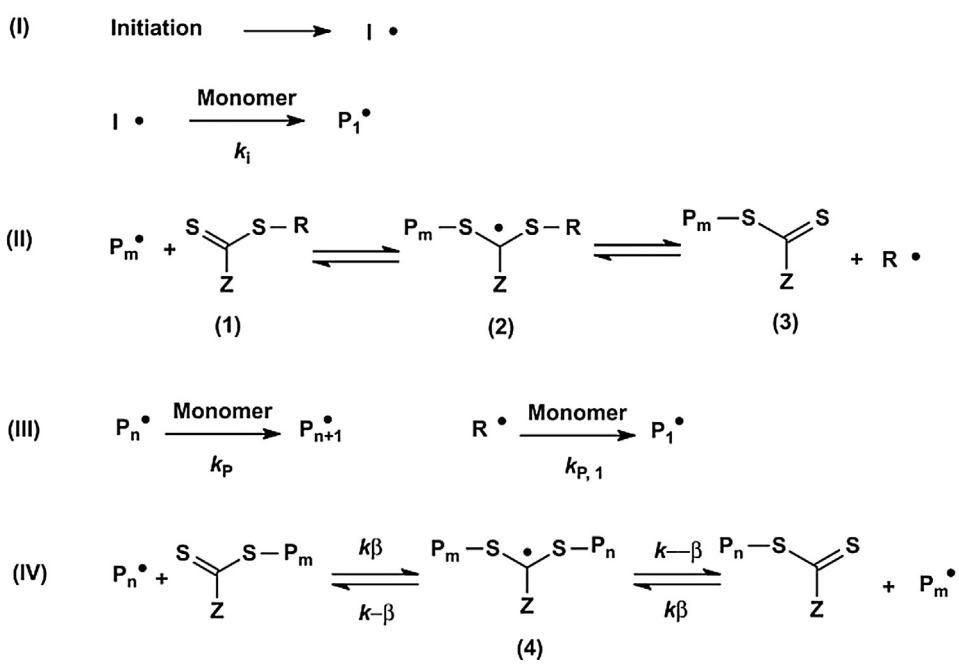

Fig. 5. Mechanism of a RAFT polymerization. The CTA plays the role of activator/deactivator. Reproduced from Ref. [86], Copyright(2002), with permission of John Wiley and Sons.

produced (see Sections 3.2.1 and 3.2.2). If free polymer is generated, the molecular weight can be easily determined by size-exclusion chromatography (SEC) or viscosimetry. From optical waveguide lightmode spectroscopy (OWLS) the mass of a deposited polymer can also be obtained [23].

Moreover, the morphology, graft density and thickness have to be determined directly on the surface. AFM is a powerful tool to study morphology [103,104] (Fig. 6). For example, in the case of PNIPAM, a change in conformation accompanying a change in temperature can be observed by AFM [103].

Surface plasmon resonance (SPR) gives the wet thickness of the layer, *i.e.* its thickness in a liquid environment, taking into account swelling phenomena. The dry thickness, *i.e.* in the absence of water, of the grafted layer can be measured by ellipsometry [103]. AFM can also be used to determine the dry thickness, but systematic errors were reported due to AFM tips being attracted by the PNIPAM layer [105]. PNIPAM grafting density can be deduced from the dry thickness value, through the following equation:

$$\sigma = \frac{h\rho N_A}{M_n}$$

with σ the graft density, h the dry thickness, N_A Avogadro's number and M_n the molecular weight. The density of dry PNIPAM, ρ, is sometimes arbitrarily taken equal to 0.95 g/cm^3 by certain authors [106], but the actual density can be measured by U-tube oscillation [107] or by X-ray reflectometry [108]. Fourier transform infrared (FTIR) spectroscopy can also be used, in some cases, to determine the graft density, as reported by Mizutani *et al.* [109].

Chemical analysis can be performed through classic surface analysis: X-ray photoelectron spectrometry (XPS), attenuated total reflection FTIR spectroscopy (ATR-FTIR), Raman spectroscopy. For nanolayer studies, XPS is preferable to ATR-FTIR spectroscopy due to its lower penetration depth (a few nm for XPS against up to 1 μm for ATR-FTIR spectroscopy) [28]. Secondary ion mass spectroscopy (SIMS) techniques can be even more surface localized. In this technique, the surface is etched by an ion beam and sputtered material is collected by a detector. Elements and chemical structure can be determined.

Quartz microbalance (QCM) is an interesting tool for the evaluation of the amount of a deposited layer. The determination of the quantity of proteins adsorbed on a biomaterial surface can be obtained by measuring, *in situ*, the frequency shift of the quartz

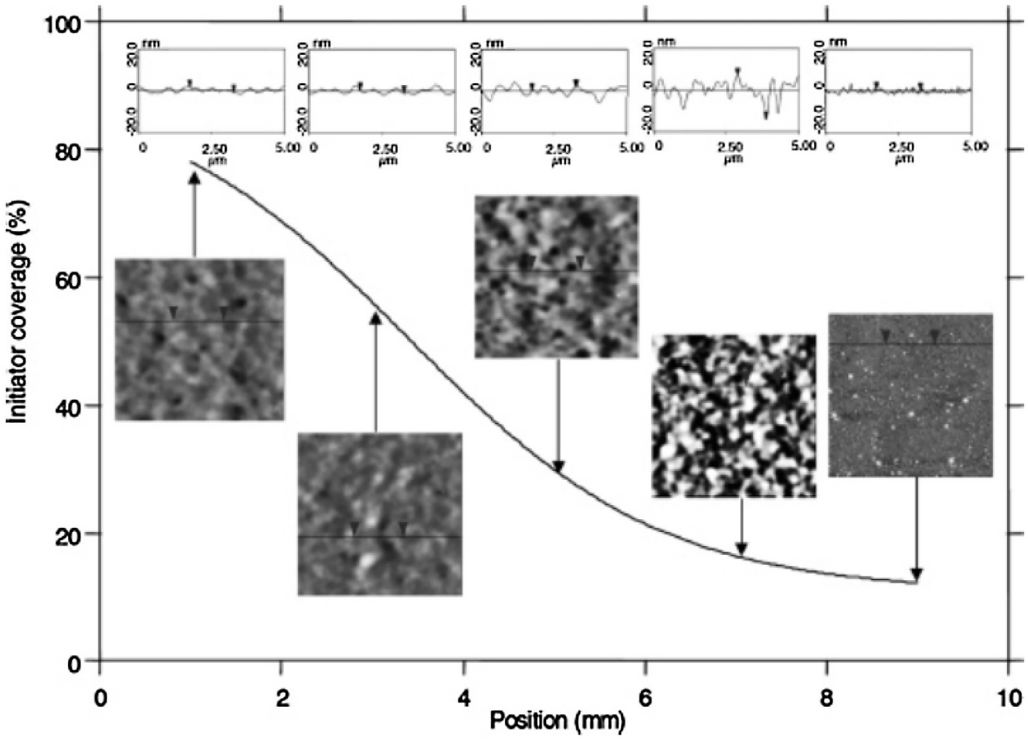

Fig. 6. Relationship of PNIPAM film morphology to local grafting density as tracked through the initiator density. The grafting density increases, from a discontinuous mushroom structure (left, low grafting density), to a heterogeneous patchy structure (middle, intermediate grafting density). At high grafting density (right), a smoother, presumably more extended, structure is obtained. Reprinted from Ref. [102]. Copyright (2006), American Chemical Society.

[24]. Quartz microbalance with dissipation monitoring (QCM-D) is useful to obtain the swelling behavior of polymer brushes [97].

Finally, carbon fibre microelectrode amperometry (CMFA) can give information about biochemical exocytosis of cells (kinetics and amount of release) and thus evaluate how the substrate impacts the excretion activity of cells [10].

Most of these techniques are not applicable to "brush grafted on polymer" systems due to the relatively high roughness of this type of surface, which explains the lack of reliable brush characterization techniques in the literature.

4. Smart bio-surfaces: example of poly(*N*-isopropylacrylamide) thermosensitive surfaces

Poly(*N*-isopropylacrylamide) is a thermo-responsive polymer. Its structure is shown in Fig. 3. Indeed, PNIPAM changes its water affinity according to the temperature of the surrounding medium, turning from hydrophilic to hydrophobic. This change occurs at around 32 °C, whose temperature is called the lower critical solution temperature (LCST), and leads to a change in its conformation. Above this temperature, PNIPAM collapses in solution. This coil to globule transition is endothermic and is related to water/polymer and polymer inter and intra-molecule hydrogen bonding. In other words, below the LCST PNIPAM is bound to water through amide/water (C=O···H—O) hydrogen bonding. As the temperature increases above LCST, the polymer becomes dehydrated and amide/amine (C=O···H—N) hydrogen bonding appears [110,111]. In the case of surfaces grafted with PNIPAM, it means that a "brush" system can be turned into a "mushroom" conformation above the LCST. The LCST of PNIPAM was reported to depend on the chain length and the grafting density while remaining between room and physiological temperature making this polymer interesting for various biomedical applications [105]. Additionally, the use of PNIPAM in a copolymer system [112], as well as the presence of salts can strongly influence this LCST [113,114]. Cl^- and CH_3COO^- have a particularly strong influence as predicted by the Holfmeister series. Moreover, ion concentrations are generally low (below 0.15 M) in both culture media and body fluids, its influence thus has to be relativized. It is noteworthy to mention that proteins, if concentrated, can also affect the LCST of PNIPAM from a decrease of 2.6 °C to an increase of 1.5 °C, depending on the protein involved [111].

Surface interactions can be modulated by the temperature of the PNIPAM. This variation of the interactions is clearly observed by AFM measurements [105,115]. Bovine serum albumin (BSA) bound to AFM tips was used to study the variation of protein/PNIPAM surface interactions. It appeared that interactions are temperature dependent, with a protein adsorption phenomenon occurring above LCST [116]. This phenomenon was also observed using a QCM. The mechanism is related to PNIPAM hydration but is not well understood at the present time. Various studies were performed to evaluate the ability of PNIPAM to trigger cell attachment. In addition to their slight control of LCST, it appears that the grafting density and the chain length also play an important role on bioadhesion. Thus, the material seems to be resistant to the adsorption of either proteins or cells when chain density and chain length are both high [117]. This is explained by the difficulty for proteins to enter the PNIPAM layer due to steric hindrance when chain grafting is dense. Halperin *et al.* proposed a theoretical approach of mechanisms for harvesting cells cultured on thermoresponsive PNIPAM polymer brushes [118].

First, two interaction modes have to be examined when we consider particles (*e.g.* proteins) and a brush structure. The compressive mode, where the brushes are compressed by a particle, occurs when its size is greater than the space available between chains, taking into account the ability of chains to rearrange themselves around the particle. This is typically the case for cells. The other mode, the insertive mode, occurs for small particles, *e.g.*

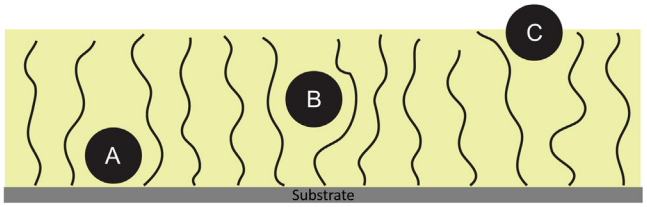

Fig. 7. The three different modes of protein adsorption through brushes: (A) primary adsorption, (B) ternary adsorption and (C) secondary adsorption. Adapted from Ref. [117], Copyright(2012), with permission of Elsevier.

extra cellular proteins such as fibronectin. Here, we see the importance of the grafting density: high density limits protein insertion. Then, the depth of the inserted protein can also vary, forming three modes: primary, secondary and ternary adsorption (Fig. 7). High affinity between ECM proteins and substrate promotes primary adsorption, whereas a high grafting density tends to suppress primary and maybe ternary adsorption [117,118]. Primary and ternary adsorption favor bioadhesion, whereas a theoretical model predicts that secondary adsorption preferably occurs for large cylindrical proteins [44,119]. Nonetheless, if ternary adsorption mediated bioadhesion was shown to be possible, its effect on protein denaturation is still to be demonstrated [118]. Finally, a thin layer (*i.e.* low molecular weight) facilitates primary and ternary adsorbed proteins/cells interactions, leading to an increase of cell adhesion. In addition, protein adsorption is also concentration dependent and, at high concentrations, the adsorption rate below and above LCST can become close [120]. Then, the graft density was shown to influence the brush structure. A high graft density can cause phase separation [121], and more generally the density will modulate the protein adsorption rate within the brushes, as for BSA [44]. Malham *et al.* showed that chain rearrangements over time could slightly increase inter-chain adhesion and related this to –NH and C=O hydrogen bonding [121]. It is obvious that inter-chain attractive interactions would play a role on protein inclusion.

Various cells adhere to heated PNIPAM surfaces and are released during cooling [122]. Typically, cell detachment is achieved at T = 20 °C [118]. This property allows cells to be seeded and gently detached them, without a need of trypsin: it is used in the field of cell sheet engineering [123].

Mono or multilayer cell sheets are thus produced and used for tissue regeneration [2]. These biological layers demonstrate good integration in tissues. I*n vivo* studies were performed to treat various diseases: cartilage degeneration [124], damaged corneal tissues [125] or cardiac tissues [126]. Interestingly, it appeared that after cell lift-off, a layer of ECM remains attached to the surface. Research has tried to determine the composition of this remnant protein layer [127] and it was shown that most of the fibronectin leaves the surface with the cells. Nonetheless, this remnant layer promotes new cell growth, showing its viability.

A lot of systems have already been developed using PNIPAM brush surfaces. PNIPAM brushes were successfully grafted through ATRP [69,128] and RAFT polymerization [90,93]. By introducing reactive groups through plasma treatments, PNIPAM can be grafted *via* "grafting to" amide binding [8] or surface initiated ATRP [81]. ATRP produced Si-PNIPAM brush hybrids which were shown to be efficient in the thermo-triggered adhesion/de-adhesion of fibroblast cells [128]. In this case the thicker the PNIPAM layer is, the more profitable the surface is for cell proliferation after 2 days. In all cases, no adhesion is observed for temperatures below LCST independently of the thickness (3 nm, 11 nm or 31 nm). It also appeared that the antifouling properties of the poly(ethylene glycol) monomethacrylate (PEGMA) in combination with PNIPAM increases the cell release abilities of PNIPAM. For bovine endothelial cells, a thickness of PNIPAM brush on tissue culture polystyrene around 15 nm showed optimal adhesion/de-adhesion properties [129]. They also reported no adhesion above 30 nm, whereas Mitzutani *et al.* observed that endothelial cell adhesion on polystyrene ATRP grafted PNIPAM surfaces is suppressed for thickness greater than 60 nm [109]. Moreover, the best adhesion was obtained for thinner PNIPAM layers (1.8 nm). Takahashi *et al.* developed surface-initiated RAFT polymerization brushes on glass coverslips and studied both graft density and molecular weight of PNIPAM on reversible bioadhesion [90]. The study showed that the amount of cells also increased on lowering the graft density. In addition, better bioadhesion is observed for shorter brushes but de-adhesion needs a thick enough layer. The explanation is related to the necessity to push cells from the surface, as the PNIPAM brushes become extended on reducing the temperature. This can be the general conclusion, if possible, of thickness considerations: a balance between the ability for cells to attach (thin brush layer) and detach (thick brush layer) as to be found. Consequently, a thick PNIPAM layer can be useful to produce protein resistant surfaces.

Zhao *et al.* studied the anti-fouling properties of PNIPAM grafted polyurethane surfaces against fibrinogen and human serum albumin (HAS) proteins at 37 °C [131]. It appeared that the thermosensitivity of the hydrophilicity was not significant on low PNIPAM thickness, and that the protein adsorption strongly decreased as this thickness increased. This effect can be due to higher hydrophilicity of thicker layers. As a consequence, cells do not adhere to thick brushes and thus anti-adhesion surfaces can be produced by the use of PNIPAM. Yu *et al.* showed thickness dependent thermo-sensitivity of PNIPAM grafted Si (surface initiated ATRP) surfaces and managed to produce HSA repellent, even with thin PNIPAM layers (< 15 nm) [132]. The variation of contact angle and HSA adsorption between 27 and 37 °C is not so notable at low PNIPAM thickness. However, greater temperature sensitivity was observed at higher graft thickness both on contact angle and HSA protein adsorption. More interestingly, at 37 °C HSA adsorption is not linearly dependent with PNIPAM thickness and, as the thickness increases, a decrease of sensitivity follows. This observation was attributed to possible adsorption on the Si-initiator surface at low graft thickness. As contact angle showed hydrophobic surfaces (higher than traditional anti-fouling polymer), the authors deduced that the anti-fouling properties of low PNIPAM thickness were not due to the hydrophilicity of the PNIPAM surface, but to the interactions between PNIPAM and the substrate. Indeed, short PNIPAM brush end chains can also interact with the substrate and reduce the freedom of conformation changes, reducing the temperature effect [132]. This study also showed the importance of the protein size on adsorption. Indeed, the size of the protein molecule is of importance as the penetration will be dependent on steric hindrance phenomena. In addition, it can be noted that the three proteins studied, HSA, fibrinogen protein and lysozyme also have different charge characteristics. The smallest protein, the lysozymes, adsorbed whether or not the PNIPAM was in collapsed or extended regime. An explanation can be the ability of this small protein to pass through the PNIPAM brushes and then to interact with the substrate (primary adsorption). As the protein size increased, the proteins were no longer able to efficiently go through PNIPAM chains below the LCST (extended regime), but are able, above the LCST, to interact with the outermost region of PNIPAM when hydrophobic and maybe with the substrate (collapsed regime).

Comparing these two last results, it appears that in the case of polyurethane substrate the hydrophilicity tends to increase with the thickness, leading to a decrease of protein adsorption [131], whereas the Si substrate graft led to an increase of protein adsorption, as the hydrophobicity increased [132]. Thus, we see the importance of the substrate, and the resulting surface properties will depend on the ability of its substrate to allow primary binding and on the hydrophilicity/hydrophobicity balance of the resulting

surface. In fact, adsorption of HSA was of the same order of magnitude whatever the substrate, for thicker PNIPAM layers, the latter having also the same hydrophilicity.

Nanostructured or patterned surfaces were also investigated. Silicon nanowires were thus used as a substrate for SI-ATRP [133]. The addition of PNIPAM strongly reduced platelet activation and adhesion, both above and below the LCST. As expected, the nanostructuration of PNIPAM surfaces (*i.e.* the increase of surface area) involves an exacerbation of the hydrophilic/hydrophobic surface state. In fact, Chen *et al.* highlighted that these nanostructures, which present a high aspect ratio, tend to trap water. This entrapped water led to a reduction of the platelet protein/surface interactions, whatever the coil or globule state of PNIPAM brushes. These results open new fields of application as platelet activation and adhesion can lead to blood coagulation and thrombosis. More recently, silicon nanopillars were shown to be able to reversibly attach/detach to/from breast cancer cells, through specific and selective interactions [134]. Compared to flat Si-PNIPAM surfaces, the introduction of nanopillar architecture widened the overall potential contact surface but limited the available space for interactions between cells and surfaces when adhered. As a result, the 3D architecture of these surfaces enhanced cell capture, but diminished the tendency of cells to spread, making release easier. Nanopatterned PNIPAM surfaces were also used to trap, kill and deliver bacteria [135]. In this work, biocides were grafted between patterned SI-ATRP PNIPAM brushes. Additionally, nanopatterning is a potential solution to overcome thickness limitations: even thick brushes allows cells to attach, so the necessity to have thick enough brushes in order to detach cells can be more easily fulfilled [136]. Owing to the fact that thick PNIPAM brushes do not support cell attachment but become bioadhesive when nanopatterned, controlled spatialization of cell culture is possible [136].

In the field of body implants and surgical biomaterials, Chen *et al.* grafted ("grafting to") PNIPAM-COOH onto chitosan through an amide bond resulting in a comb-like polymer structure (branched PNIPAM on a chitosan backbone) which forms a gel [137]. The surface functionalization was followed by a study of chondrocytes and meniscus cells bioadhesion. The thermosensitive behavior of PNIPAM (brush to mushroom thermo triggered conformation change) was shown to provoke a phase transition, liquid to solid-like hydrogel. The gelification would occur inside the body after injection. Fibronectin adsorption was observed by fluorescence using rhodamine labelled fibronectin. Polypropylene-g-chitosan-g-PNIPAM was performed through a "grafting to" process with a view to easily stripping off of the skin wound dressing [138]. Non-toxicity and temperature-responsiveness behavior were fulfilled.

While brushes do present some interesting properties, other non-brush systems have been used to develop the same kind of functionalities. Ignacio *et al.* made a wound dressing using UV grafted PNIPAM polyurethane membranes [139]. New subcutaneous connective tissue grew but no toxicity was observed. The detachment on mice skin wounds was triggered by the reduction of temperature below the LCST. We can also mention the easy removal of retinal implants achieved with PNIPAM surfaces [140]. In this study, bioadhesion, measured by a pull-off test, appeared one minute after passing through the LCST. The correlation between the cell culture behavior and thermo-sensitive tissue adhesion clearly indicates that bioadhesion on tissues is related to the ability of PNIPAM to adsorb proteins and thus catch cells.

5. Conclusion

Bioadhesion is a characteristic of interactions between materials and cells. This phenomenon is now better understood and gives rise to interesting fields in biomedical science such as cell sheet engineering. Brush structures have been shown to be efficient for cell adhesion, and to offer the advantage of a well-controlled preparation process. The "grafting from" approach enables dense brush layers to be made without steric hindrance limitations and thus leads to homogeneous layers, especially for rough surfaces. Living polymerization provides a way to control the growth of these layers. Various techniques exist, such as Fe-catalyzed ARGET-ATRP or RAFT polymerizations. It is known that the grafting method can lead to different properties (*e.g.* cross-linking), and impact the surface interaction with cells. PNIPAM, as a thermo-sensitive polymer, is widely studied and is a promising polymer in the cell sheet generation area, but its applications can be wider, including, for example, implants. Thus, several parameters such as graft density, layer thickness and grafting method have to be studied, characterized and compared in terms of cytotoxicity and bioadhesion. As yet, no solutions have been found to thoroughly characterize and study brushes directly on polymer substrates, this challenge will have to be overcome in the future. This limitation puts a brake on the control of the surface state, which is a key point for the prospective work in bioadhesion and can allow the investigation of new insights in the bioadhesion field, both theoretically and in terms of applications. In some cases, (*i.e.* biomedical implants) antifouling surfaces are sought in order to limit the biological colonization or any immunological response. On the contrary, tissue engineering needs good integration, and thus bioadhesion, of cells within biomaterials. Strong efforts are needed to further investigate the effects of the physio-chemical parameters of surfaces: hydrophilicity, roughness, mechanical properties, patterns. The development of innovative biomaterials will be dependant of these advances.

Acknowledgement

Authors would like to thank the French National Research Agency for its financial support (ANR-14-CE17-0002-01, FP-BioPrev project).

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