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Xyloglucan-Derivatized Films for the Culture of Adherent Cells and Their Thermocontrolled Detachment: A Promising Alternative to Cells Sensitive to Protease Treatment

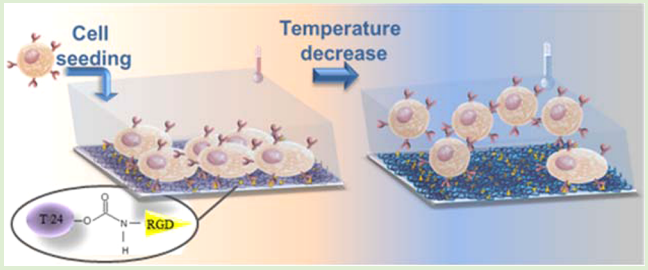
Amanda K. A. Silva,[†,‡,∥] Cyrille Richard,[†] Guylaine Ducouret,[§] Michel Bessodes,[†] Daniel Scherman,[†] and Otto-Wilhelm Merten*[,‡]

[†]Unité de Pharmacologie Chimique et Génétique et d'Imagerie; CNRS, UMR 8151, Paris, F-75270 cedex France; Inserm, U1022, Paris, F-75270 cedex France; Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Sorbonne Paris Cité, Paris, F-75270 cedex France; and ENSCP, Paris, F-75231 cedex France, Chimie Paritech, France

[‡]Genethon, 1 bis rue de l'Internationale, BP 60, 91002 Evry cedex, France

[§]Physico-chimie des Polymères et des Milieux Dispersés, UMR 7615, UPMC−CNRS-ESPCI, 10 rue Vauquelin, 75231 Paris cedex 05, France

ABSTRACT: By taking advantage of a natural and abundant polymer as well as a straightforward film formation technique, this paper focuses on the conception and use of a new alternative tool for thermocontrolled cell detachment. Thermoresponsive xyloglucan was produced after partial galactose removal by a 24 h reaction with β-galactosidase. The obtained polymer (T24) was then activated by reaction with 4-nitrophenyl chloroformate (NPC) in order to graft a cyclic peptide presenting an arginine-glycine-aspartic acid (RGD) motif. The effect of RGD grafting on the sol−gel transition temperature of T24 is evaluated by rheological measurements. Solvent-casted films of T24-RGD successfully promoted cell adhesion, proliferation, and thermocontrolled detachment. The presented approach is a new alternative for cells sensitive to the proteolytic treatment routinely used for cell detachment. Because the RGD sequence used herein is widely recognized by different cell types, this protocol may be extended to other cells. Besides, the presented chemical route can be applied to different peptide sequences.



INTRODUCTION

The optimization of cell amplification protocols is a key point in biotechnology. In this context, the detachment of adherent cells is an important step. Enzymatic detachment represents the most widespread method for adherent cell dissociation. It consists in the use of enzymes such as collagenase, dispase, or trypsin. Although enzymatic methods readily enable large-scale expansion, they may result in cell damage. Indeed, this approach was observed to be quite inadequate for detaching some cells, such as microglia, because this treatment altered their metabolism and cellular responses. Two possible reasons for these alterations are considered. One possibility is that the enzymatic method may select certain subpopulations during harvest. If only specific subpopulations are recovered, changes in cellular properties and responses in these populations would not be unexpected. The other possibility is that enzyme digestion alters signal transduction and gene expression by forcing transduction through inappropriate signaling pathways.[1] Besides, emerging evidence suggests that such techniques are associated with increased rates of karyotype abnormalities in the case of human embryonic stem cells (HESC).[2,3]

An alternative for the enzymatic method is the use of mechanical ones. They avoid enzymes and seem to maintain genetic stability. In addition to that, mechanical detachment allows selective transfer of exclusively undifferentiated colonies, considering HESC. However, the comparatively laborious and time-consuming process of manual colony dissection limits the practical use of mechanical passaging in bulk cell culture.[4] Therefore, innovative approaches are requested to overcome the presented drawbacks related to cell passaging.

Developing stimuli-responsive polymers as "intelligent" cell culture surfaces may represent an interesting possibility. Stimuli-responsive polymers are defined as polymers that undergo relatively large and abrupt changes in response to external stimuli, which can be chemical (such as pH or divalent cation concentration) or physical (such as temperature).[5,6] In culture substrates based on stimuli-responsive polymers, cell detachment can take place by adjusting such physical or chemical parameters, instead of using mechanical or enzymatic methods. This approach has been widely studied using poly(*N*-isopropylacrylamide) (pNIPAM) as a thermoresponsive polymer.[7−11] In some studies, pNIPAM-based surface treat-

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ment was carried out by solvent casting[12−14] or living radical polymerization.[15−17] In other ones, pNIPAm's poor cell affinity has been counterbalanced using costly and complex grafting techniques for ultrathin film formation.[18]

The evaluation of natural abundant polymers combined to straightforward techniques of film formation, such as solvent casting, for temperature-assisted cell detachment has not been appropriately addressed. Herein, we propose xyloglucan-derivatized films as a new alternative for the culture of adherent cells and their temperature-assisted detachment. Xyloglucan is the major hemicellulose component of primary walls of many higher plants. This polysaccharide has a backbone consisting of glucose residues, with side groups of xylose attached through glycosidic linkage. Some xylose residues are additionally substituted with galactose or the disaccharide galactose and fucose.[19,20] Considering the abundance of this polymer and its properties, xyloglucan may be a promising polymer for the development of culture surface. Indeed, the use of xyloglucan as a cell-supporting matrix has already been proposed.[21] However, in such a case, the interest in the polymer related to its galactose moieties for hepatocyte attachment.

Xyloglucan can be rendered thermally responsive by using fungal β-galactosidase to remove more than 35% of the galactose residues. For instance, considering a galatose removal ratio of 35%, xyloglucan becomes gel on heating at 40 °C.[22] The thermosensitive property of xyloglucan has been mainly exploited in pharmaceutical technology in the development of oral,[23] rectal,[24] and nasal[25] delivery of drugs and for intraperitoneal injections.[26] The sustained release of drugs by xyloglucan hydrogels has been reported.[27]

When developing cell culture surfaces, cellular adhesion to the support is the main issue. In biological tissues, most of the cells require adhesion to an extracellular matrix (ECM) for survival and growth. Cell−matrix adherent junctions enable cells to bind the ECM by connecting the actin filaments of their cytoskeleton to the matrix.[28,29] Members of a large family of cell−surface matrix receptors called integrins mediate this adhesion. The tripeptide sequence Arg-Gly-Asp (RGD) has been identified as part of many natural integrin ligands.[28] In fact, the RGD sequence has been identified as cell attachment site for a large number of adhesive extracellular matrix, blood, and cell surface proteins. Nearly half of the over 20 known integrins recognize this sequence in their adhesion protein ligands. Some other integrins bind to related sequences. A partial list of adhesion proteins with RGD sites includes fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin, entactin, tenascin, osteopontin, and bone sialoprotein.[30]

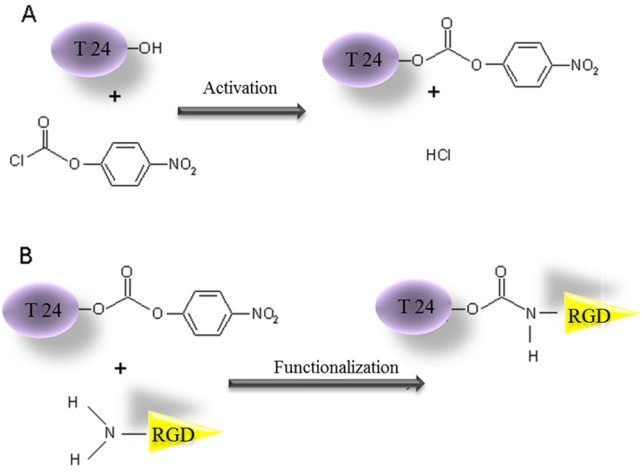
The present study concerns the development of a temperature-responsive culture film based on partially degalactosylated xyloglucan, which was further chemically modified by RGD groups in order to promote cell adhesion and proliferation.

MATERIALS AND METHODS

β-Galactosidase Reaction. The production of thermoresponsive xyloglucan at a galactose removal ratio (GRR) of 40% was carried out as reported previously,[31] by reacting 1 g of xyloglucan from *Tamarindus indica* (Megazyme International, Ireland) in 50 mL of aqueous medium with 31.35 mg of β-galactosidase from *Aspergillus oryzae* (Sigma, U.S.A.) at 11.8 units/mg. Briefly, the reaction was carried at 53 ± 2 °C during 24 h. The sample was then heated at 100 °C for 20 min to inactivate the enzyme. The partially degalactosylated xyloglucan was precipitated from this solution by addition of ethanol. After three cycles of dispersion in water followed by precipitation in ethanol, the product was dried at 60 °C over a period of two days. This sample is thereafter identified as T24. The supernatant obtained after purification was analyzed to confirm the GRR using an enzymatic kit [Lactose/D-Galactose (Rapid) Assay Kit − Megazyme International Ireland Ltd. (Ireland)]. The GRR ratio was determined as (released galactose residues × 100)/total galactose residues. The amount of total galactose residues was measured after total hydrolysis by heating the polymer with 2 N sulfuric acid at 100 °C for 3 h.[22]

RGD Grafting on Partially Degalactosylated Xyloglucan. After the enzymatic reaction, some remaining hydroxyl groups of the polymer were reacted with *p*-nitrophenylchlorocarbonate (NPC)[32] to achieve an activated material able to react with the RGD peptide (Scheme 1). The T24 preparation (600 mg) was solubilized in 40 mL of dimethylsulfoxide (DMSO, Sigma, Germany) at 50 °C over a period of 3 h. Then, 2.4 g 4-nitrophenyl chloroformate (Sigma, Germany) was added, followed by 40 mL of pyridine (SDS, France) and 240 mg 4-dimethylaminopyridine (DMAP, Fluka, Switzerland). After overnight stirring at room temperature, the reaction product was precipitated with ethanol (50 mL) and recovered. This process was repeated three times. The degree of activation was determined spectrophotometrically by measuring, at 402 nm (ε = 18400 cm^{-1} M^{-1}) with a UV−visible Cary 100 spectrophotometer (Varian, U.S.A.), the amount of *p*-nitrophenylate released after reaction with NaOH (2 N).[33] We used 1 mL of NaOH (2 N) per mg of product. The activated T24 was stirred in 75 mL of DMSO at 50 °C during 3 h, and after its solubilization, 120 mg of DFKRG cyclic peptide (American Peptide, U.S.A.), 1.7 mL of triethylamine (SDS, France), and 1.47 g of DMAP (Fluka, Switzerland) were added. The reaction was then allowed to proceed overnight under stirring at room temperature and was followed by the inactivation of unreacted nitrophenylcarbonate groups with 600 mL of triethylammonium acetate buffer at 60 °C during 4 days. Amino acid analysis was carried out to evaluate the amount of grafted peptide using an Aminotac JLC500/V equipment (Jeol, Tokyo, Japon). For this purpose, 2 mg of T24-RGD was reacted with 6 M HCl at 110 °C during 24 h in order to cleave peptide bonds. The result was also qualitatively confirmed by fluorescence spectrophotometry by grafting a FITC-DFKRG peptide (American Peptide, U.S.A.) to T24. The purified product was solubilized in water (1 mg/mL) and the fluorescence intensity of this solution was obtained with a Cary Eclipse fluorescence spectrophotometer (Varian, U.S.A.). Excitation and emission wavelengths were 488 and 515 nm, respectively. Quantification was performed by means of a calibration curve from standard FITC-

Scheme 1. RGD Functionalization of Partially Degalactosylated Xyloglucan (T24)[a]



[a](A) Activation of T24 hydroxyl groups by 4-nitrophenyl chloroformate. (B) Reaction of activated T24 groups with cyclic DFKRG (RGD).

DFKRG solutions in water at concentrations ranging from 90 to 2 nmol/mL.

Rheology. The thermogelation properties of native xyloglucan (T0), T24, and T24-RGD were evaluated by rheology (Figure 1). The

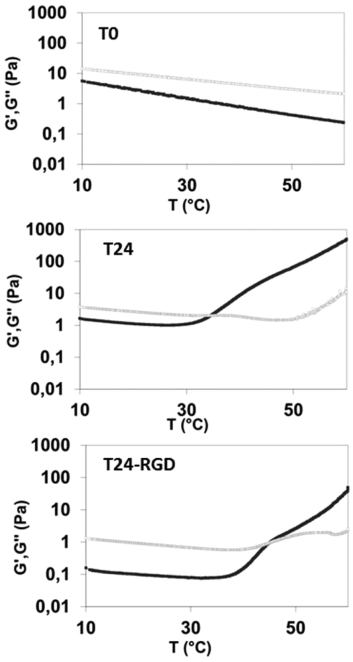


Figure 1. Temperature-dependence of the storage modulus G' (in black color) and the loss modulus G'' (in gray color) for autoclaved T0, T24, and T24-RGD in PBS at 2% (w/v).

oscillatory shear measurements were performed on a stress-controlled rheometer RS600 Haake (Germany) equipped with a cone-and-plate geometry (35 mm diameter, 2° angle, and gap size of 50 μm). The values of the stress amplitude were checked to ensure that all measurements were conducted within the linear viscoelastic regime, where the dynamic storage modulus (G') and loss modulus (G'') are independent of the stress amplitude. The measuring unit was equipped with a temperature unit (Peltier plate) that provided temperature control during analysis. Rheological behavior of the sol−gel transition was measured by performing temperature sweeps. Temperature-dependence of storage (G') and loss (G'') moduli were observed by heating the systems from 10 to 60 °C at a rate of 0.5 °C/min. The samples were protected by a homemade cover to prevent water evaporation. Samples were prepared by dissolving 200 mg of T0, T24, or T24-RGD in PBS (10 mL) and stirring at 4 °C for two days. The obtained solutions were stored at 4 °C.

Cell Culture Studies. A375 cells (ATCC #CRL-1619), over-expressing the RGD receptor, were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, U.S.A.) supplemented with 10% of fetal calf serum (FCS, PAA, Austria) on 75 cm^2 culture flasks (TPP, Switzerland). Cell studies were performed in 24-well plates (TPP, Switzerland) previously coated with autoclaved T24 or T24-RGD dispersed in PBS. Coating was carried out by adding 0.6 mL of each polymer and drying for 2h at 60 °C under vacuum. By using this protocol, 2-mm thick stable films coated the wells. Drying step was necessary for obtaining stable films. Otherwise, the polymer layer dissolved after the addition of the cell culture medium even at 37 °C. Coated wells were incubated with culture medium during 24h, and the medium was changed three-times during this period. For T24, a concentration of 3% was used, whereas for T24RGD, a mixture (1:1 vol/vol) of this hydrogel at 5% with T24 at 3% was used. Uncoated wells were used as a positive control. Cells were seeded at a density of 20.000 per well and incubated at 37 °C under a humidified atmosphere of 5% CO_2 in air. The culture medium changes and the cell passages were carried out twice and once a week, respectively. Cell adhesion on T24- and T24-RGD-coated wells and uncoated wells was observed by fluorescence microscopy using an Axiovert 135 microscope (Zeiss, Germany) and carboxyfluorescein succinimidyl ester (CFSE, Sigma, Germany) to label cells (Figure 2).

For temperature-assisted cell detachment (Figure 3), the culture medium was replaced by 1 mL of a fresh one at 4 °C and the plate was

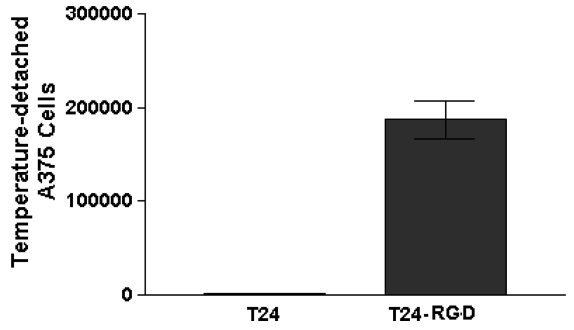


Figure 3. Temperature-assisted cell detachment from T24- and T24-RGD-coated wells after 7 days of culture (20.000 A375 cells were seeded per well; 24-well plate).

incubated for 1 h at room temperature. For a kinetics experiment, cell detachment was evaluated at 30, 60, 120, and 240 min at 4, 20, and 28 °C (Figure 4). The number of cells detached from the T24- and T24-RGD-coated wells was evaluated by hematimeter cell counting. The number of cells in the uncoated wells was also determined by hematimeter cell counting after trypsinization. After a three-week culture on T24-RGD-coated and uncoated wells, 20.000 cells of each condition were seeded on uncoated wells, incubated overnight and cell metabolic activity was evaluated by spectophotometry using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Germany; MTT test; Figure 5). In brief, 5 mL of MTT solution at 5 mg/mL was diluted with 45 mL of culture medium and 1 mL of such solution was added to each well. The plate was incubated at 37 °C during 3 h, the supernatant was carefully aspirated and 1 mL of lysis

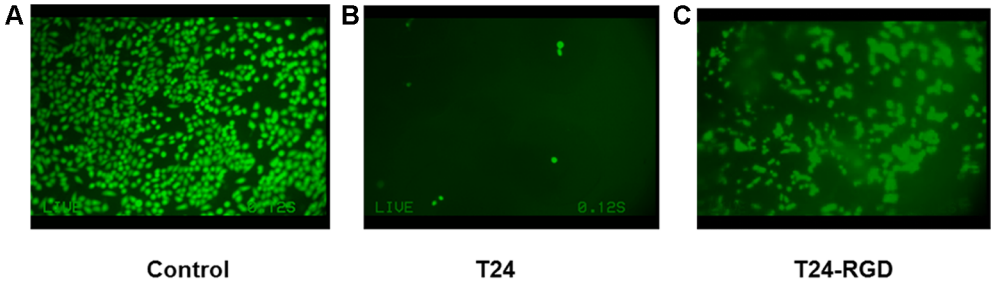


Figure 2. Attachment of A375 cells on three types of support: (A) polystyrene plate; (B) T24-coated polystyrene plate; (C) T24-RGD-coated polystyrene plate. Images of wells two days after cell seeding at 100× magnification (seeding cell number: 20.000 cells per well; 24-well plate).

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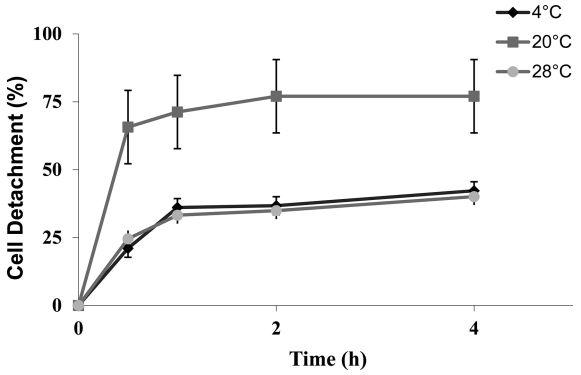

Figure 4. Cell detachment kinetics from T24-RGD films after 7 days of culture (the amount of cells detached by trypsinization was considered as 100% cell detachment).

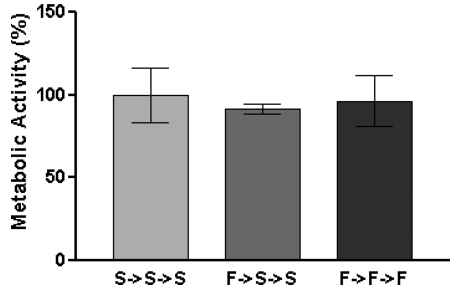

Figure 5. Metabolic activity of A375 cells after a 3 week culture. S→S→S stands for cells that underwent three passages on standard uncoated wells using trypsinization for cell detachment (control). F→F→F stands for cells that underwent three passages on T24-RGD films using the temperature-assisted method for cell detachment. F→S→S stands for cells that underwent one passage on T24-RGD films using the temperature-assisted method for cell detachment and two passages on standard uncoated wells using trypsinization for cell detachment. Data are normalized to the control level of cell metabolic activity.

solution (10 g of Triton X-100 and 10 mL of 1 N HCl in 100 mL of anhydrous isopropanol) was added to each well. The optical density was determined at a wavelength of 562 nm.

MTT test was also used to evaluate the effect on the metabolic activity of our temperature-assisted detachment method (Figure 6) and effect of cell exposure to different concentrations of T24 and T24-RGD in solution (Figure 7). Cell proliferation on uncoated standard wells during three weeks after a first passage on T24-RGD-coated wells was also assessed (Figure 8).

Data Analysis. Data are presented as one standard deviation from the mean (n = 3). The Student t-test was performed to determine a

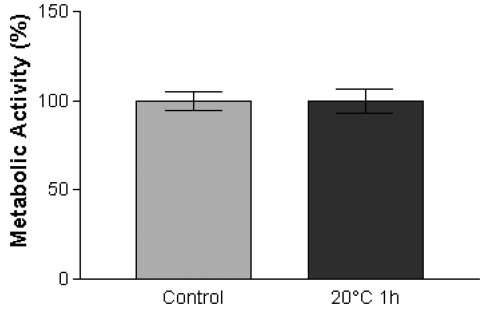

Figure 6. Metabolic activity of A375 cells plated on standard uncoated wells either nontreated (control) or submitted to the temperature-assisted method for cell detachment (20 °C 1 h). Data are normalized to the control level of cell metabolic activity.

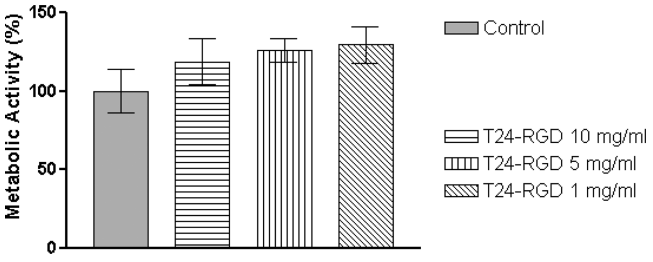

Figure 7. Metabolic activity of A375 cells incubated with 1, 5, and 10 mg/mL of soluble T24-RGD. Data are normalized to the control level of cell metabolic activity.

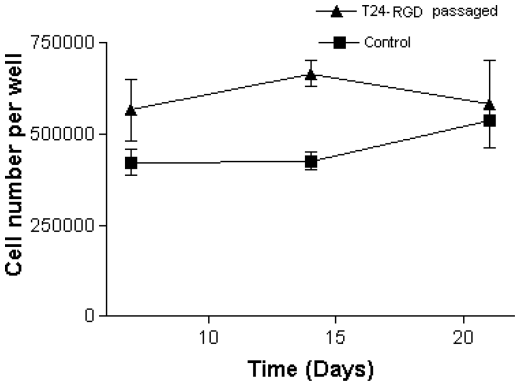

Figure 8. Cell proliferation on uncoated standard wells during three weeks after a first passage on T24-RGD-coated wells. The control was cell proliferation exclusively performed on uncoated standard wells. Cells were passaged once a week (seeding cell number: 20.000 cells per well; 24-well plate).

significant difference between the experimental and control groups using Prism 3.0 version of GraphPad software (U.S.A.). A 95% confidence level was considered significant.

RESULTS AND DISCUSSION

RGD Grafting on Partially Degalactosylated Xyloglucan. Grafting biologically active molecules on polymers is an attractive approach in biotechnology. Our strategy to functionalize T24 with a Arg-Gly-Asp (RGD) sequence involved an activation step[33] carried out using 4-nitrophenyl chloroformate (NPC) for a partial conversion of the primary hydroxyl side groups on a T24 polymer chain (Scheme 1a). After purification, hydrolysis of a small fraction of the activated polymer was performed in order to determine the activation rate. The presence of an absorption band at 402 nm corresponding to the 4-nitrophenylate confirmed the activation of T24. The concentration of this compound determined spectrometrically indicated a ratio of six activated hydroxyl groups per T24 molecule.

After T24 activation, the reaction with the RGD peptide (cyclic DFKRG) was studied. The reactive carbonate-containing T24 was reacted overnight with RGD (Scheme 1b). After the reaction, hydrolysis was carried out at pH 8 in triethylammonium acetate buffer in order to remove unreacted activated groups remaining on the polymer. After purification, the efficacy of this step was evaluated by a subsequent treatment with NaOH 0.2 M. No absorbance band at 402 nm was any more detected, thus, indicating that this procedure successfully removed unreacted activated groups.

The amount of peptide bound per mass of polymer was determined by amino acid analysis. A degree of functionaliza-

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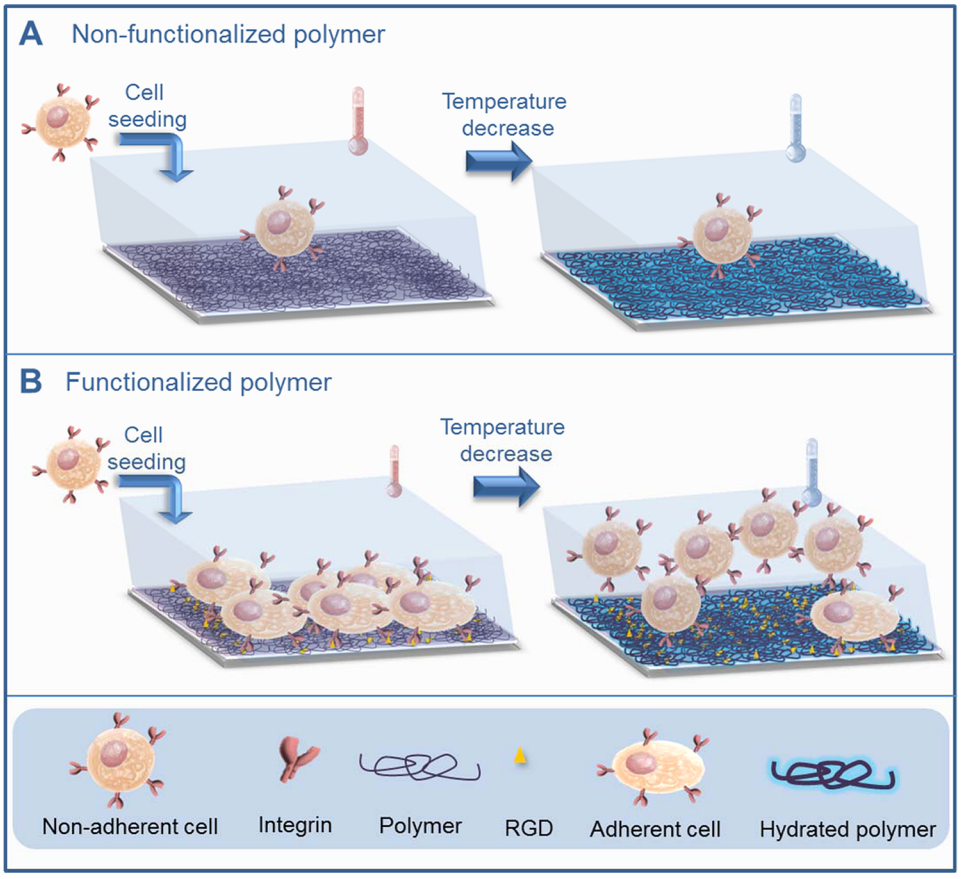
Scheme 2. Representation of Thermocontrolled Cell adhesion and Detachment on Partially Degalactosylated Xyloglucan (T24) Films: Effect of Polymer Functionalization[a]



[a](A) Poor cell adhesion and proliferation on non-functionalized films and consequent ineffective cell recover by temperature reduction. (B) Important cell adhesion and proliferation on RGD-functionalized films and high cell recover after temperature-induced detachment of cells.

tion of 27 ± 6 μmol of peptide per g of polymer was obtained. The degree of RGD grafting was also estimated by fluorescence spectrophotometry using a FITC-labeled RGD. With this second method, 14.2 ± 2.1 μmol of peptide per gram of grafted polymer was detected, a result in the same order of magnitude as obtained by the amino acid analysis of RGD grafted T24. This corresponds to a grafting ratio of about 5 RGD molecules per T24 molecule.

T24 and T24-RGD were autoclaved in order to obtain sterile samples. As reported in a previously published paper, this method, as well as γ-irradiation at 10 kGy (hydrogel irradiation in frozen state), efficiently sterilizes xyloglucan-based hydrogels and preserve the rheological properties of the polymer.[34]

Rheology. In this study, sol−gel transition evaluation was based on the temperature dependent crossover of the curves representing moduli G' and G''. According to the rheological analysis (Figure 1), the sol−gel transition for T24 and T24-RGD at 2% (w/v) took place at about 37 and 45 °C, respectively, for a heating rate of 0.5 °C/min. Native unmodified xyloglucan (T0) presents no sol−gel transition. For this sample, G' was always inferior to G'' as galactose moieties sterically hinder gelation.[22] The rheological properties of xyloglucan hydrogels at different galactose removal ratio are further discussed elsewhere.[31]

Concerning T24 and T24-RGD, both presented thermoresponsive properties. However, an increase in the sol−gel transition temperature was observed for the T24-RGD and may be attributed to a reduction in hydrophobic interactions responsible for gelation. In fact, the peptide DFKRG has a rather hydrophilic character. The peptide may act in the same way as galactose moieties in native polymer, inhibiting polymer−polymer interactions and the gelation process. Besides, DFKRG is charged at pH 7.4. As a result, it may provide both a steric and an electrostatic repulsion effect which shifts sol−gel transition to a higher temperature. In fact, it is well-known that polymer functionalization may change sol−gel transition temperature. For instance, an increase in the gelation temperature is reported for methylcellulose grafted with laminin-1,[35] while a decrease in the sol−gel transition temperature was reported for thermoresponsive polymers grafted simultaneously with both RGD and FHRRIKA, due to the presence of the relatively hydrophobic amino acids (e.g., Phe, Ile).[36]

Temperature-Controlled Cell Adhesion and Detachment. Several concentrations of T24 and T24-RGD were tested in order to obtain polymer films resistant to medium incubation. As a function of the concentration, the obtained film may undergo total dissolution at 37 °C after culture medium addition. We have obtained films resisting to culture conditions from T24/T24-RGD hydrogels at a relative concentration of 3%. This concentration was the minimum concentration used in the following experiments.

T24 and T24-RGD were evaluated for cell adhesion, proliferation and metabolic activity. The human malignant

melanoma A375 cells were chosen as model since RGD peptides can specifically bind with $\alpha_V\beta_3$ integrin overexpressed on their surface.[37] Surfaces coated with T24 or T24-RGD were initially analyzed for cell adhesion. The plates were observed by microscopy 48 h after seeding. Compared to T24-coated plates, T24-RGD-coated plates presented a much higher level of cell adhesion (Figure 2), which was attributed to the immobilized functional peptide. While 2000 ± 158 cells were recovered from the T24-coated plates, 14000 ± 912 cells were obtained from T24-RGD ones (after temperature-induced detachment), which means 10 and 70% of the seeded cells, respectively. It is likely that the $\alpha_V\beta_3$ integrins from A375 cells could recognize the RGD ligands on the surface of the film and so attach it. However, cell adhesion on uncoated wells was superior than observed for T24-RGD. This may be in part due to the soft surface of the T24-RGD layer. In fact, cell adhesiveness and spreading are enhanced by the rigidity of the substrate. Mechano-transduction across the adhesion site between cell focal adhesion plaques and the extracellular matrix attached to the substrate determines cellular fundamental behavior, including adhesion, spreading, migration, proliferation, differentiation, dedifferentiation, metastasis, and apoptosis.[38] However, this eventual drawback could be remedied by an increase in the rigidity of the T24-RGD film for instance by coupling the T24-RGD to the polystyrene support.

At day 7 after seeding, the A375 cells on uncoated wells were trypsinized. The amount of detached cells was 415000 ± 38000. A375 cells on T24- and T24-RGD-coated wells were detached by 1 h incubation at room temperature after the addition of 1 mL of fresh medium (4 °C). Compared to the number of seeded cells (20000), the amount of cells detached from T24-coated wells was markedly inferior (1300 ± 600), while an almost 10-fold increase (187000 ± 11600) was obtained from T24-RGD-coated surfaces (Figure 3). A significant difference was observed by comparing T24 and T24-RGD groups ($P < 0.0001$).

This indicates that besides being able to adhere on T24-RGD-coated plates, the cells proliferated and were also able to detach via temperature reduction without trypsinization (Scheme 2). Cell detachment by temperature reduction may be explained by surface swelling and loss of cell tension on hydrated polymer chains. Temperature-dependent swelling of ligand-attached polymer chains reduces surface rigidity. Because spread cells are in tension, a sudden loss of this tension and traction prompts detachment and rounding.[39] Besides, when temperature is decreased, thermoresponsive polymer chain hydration may extend outward, thus, shielding the RGD group from the integrins, resulting in cell detachment from the surface.[40] Peptide shielding may effectively play an important role in cell detachment, as cells are known to easily form and break integrin−RGD bonds during adhesion, due to the weak binding energy of these bonds compared to other biological bonds.[41] Additionally, it is important to highlight that the polymer partially dissolves as temperature is reduced since it is not grafted to the culture well. Although the dissolution is partial and a homogeneous polymer layer is still present on the well after 1h at 20 °C, this may markedly contribute to cell detachment. Another consequence is that detached cells are recovered in culture medium containing the polymer. Nevertheless, cell separation from the polymer in solution can be easily achieved by performing washing/centrifugation.

Concerning cell detachment kinetics (Figure 4), one can observe a two-step process independently on the incubation temperature. A fast cell detachment rate was achieved in the first 60 min. This clearly implies that temperature-induced T24-RGD hydration is a rapid process whose onset takes place before the first 30 min. The second step is best described by a remarkable change in the release slope, reflecting a reduction in the cell detachment rate. Indeed, cell detachment continued slowly onward and was not finished even after a 240-min incubation. This result correlates with earlier data on cell release kinetics from RGD-grafted pNIPAM.[40] When comparing cell detachment at 4, 20, and 28 °C, better results were obtained at 20 °C: about 30 and 70% of cell detachment, respectively, was observed after 60 min. However, at the lowest studied temperature (4 °C), the number of detached cells was smaller than at 20 °C. Such result showed that cell detachment was not directly correlated with reduced temperature and this seems to be a controversial observation. As the chains of T24-RGD are assumed to be hydrated and maintain expanded conformations at lower temperatures, resulting in reduced interactions between the cells and polymer surfaces, we should rather expect an increased detachment rate as temperature is reduced. The fact that it is not observed experimentally implies that the hydration of T24-RGD may not completely explain cell detachment from culture surfaces.

In order to interpret these results, we have to take into account the temperature effect on both polymer chains and cell metabolism. Cellular metabolic processes which actively change membrane morphology play an important role on cell detachment. As cell metabolism may be reduced by temperature, cell detachment may also be as well. Although a temperature decrease provides polymer chain hydration, the resulting reduction in cell metabolism may be a limiting factor for cell detachment. The obtained results at 4 °C are in agreement with this assumption. Related observations have been reported for the detachment of different cell types from PIPAAm grafted culture surfaces.[42]

If cell detachment was carried out by trypsinization instead of temperature-assisted method, an additional detachment of about 40% was observed, compared to the amount of detached cells after 1 h at 20 °C. Incomplete cell detachment has also been observed for other peptide-immobilized temperature-responsive surfaces based on poly(*N*-isopropylacrylamide). For instance, almost 70% of the cells may remain attached to the culture surface after 60 min of incubation at 20 °C for GRGDS-grafted pNIPAM surfaces.[41] This may be explained by an incomplete shielding of RGD peptide by the hydrated polymer resulting in a residual accessibility of the peptide sequence to integrin interaction, even at room temperature.[40]

To our knowledge, this is the first report indicating that functionalized xyloglucan-based solvent casted films successfully promote cell attachment and survival, while enabling temperature-assisted cell detachment. Reports in the literature concerning xyloglucan relate to different approaches. For instance, xyloglucan has already been functionalized with RGD and grafted to nanocellulose to support cell growth.[43] However, this material was not intended for temperature-assisted cell detachment and was produced from fully galactosylated native xyloglucan, which was treated by an endoglucanase digestion for the obtention of xyloglucooligosaccharides. A different study focused on the possibility of using thermoresponsive xyloglucan along with xyloglucan-graft-poly-D-lysine for neural tissue engineering. However, no peptide functionalization was performed nor temperature-assisted cell detachment was tested.[44]

Assessing Impact on Cell Metabolism and Proliferation. To check the stability of the metabolic activity of the cells over several passages, the temperature-assisted method was used to detach A375 cells from T24-RGD-coated wells while trypsinization was carried out for uncoated standard wells during 3 weeks (cell replating once a week). A third group consisted of A375 cells that underwent one passage from T24-RGD-coated wells using the temperature-assisted method for cell detachment and two passages from standard uncoated wells using trypsinization for cell detachment. After the third culture week, 20000 cells from T24-RGD-coated wells and uncoated standard wells were replated on uncoated standard wells and incubated overnight. Their metabolic activity was evaluated via a MTT test (Figure 5) and was similar ($P = 0.7909$). The same is true ($P = 0.3711$) for the cells transferred from T24-RGD (one-week culture) to uncoated standard wells (two-week culture). This suggests that neither the polymer itself nor the temperature-assisted method altered cell metabolic activity. Two additional experiments were carried out to confirm this hypothesis.

First, a MTT test was performed for A375 cells submitted to temperature-assisted cell detachment. This test was performed on cells plated on uncoated standard wells to set aside the effect of polymer exposure itself. Again, temperature-assisted cell detachment did not change cell metabolism ($P = 0.9799$; Figure 6). Another MTT test was performed for A375 cells exposed to T24-RGD in solution during two days in order to verify the influence of the polymer itself on the cell metabolic activity (Figure 7). Based on literature data three concentrations were studied: 1, 5, and 10 mg/mL of T24-RGD.[45] The obtained values were at or above 100%; there was no significant difference compared to control. Thus, T24-RGD can be considered as well tolerated as other thermoresponsive polymers such as poly(*N*-isopropylacrylamide), poly(*N*-vinylcaprolactam), and amphiphilic modified poly(*N*-vinylcaprolactam).[45] The lack of effect related to the polymer exposure or to the temperature-assisted method on cell metabolic activity was consistent with the results presented in Figure 5. As both were well tolerated, it is reasonable to expect a full metabolic activity for cells cultured on T24-RGD during three weeks, passaged three times by the temperature-assisted method.

To check the effect of our method on cell proliferation, cells were cultured on T24-RGD-coated wells for three weeks. The amount of detached cells by temperature-assisted method increased for T24-RGD in the subsequent culture weeks (three subsequent passages): 187000 ± 11600; 257000 ± 18480; and 330000 ± 28870 (20000 seeded cells per well at each passage). This is very encouraging and means that cell proliferation is not reduced after temperature-assisted detachment. Additionally, when A375 cells were passaged from T24-RGD-coated wells to uncoated standard wells, their proliferation was similar to that observed for cells cultured on uncoated standard wells ($P = 0.0604$; Figure 8).

CONCLUSIONS

Taken together, the obtained results demonstrated for the first time that solvent casted films of RGD-grafted T24 supported A375 attachment and survival while enabling temperature-assisted cell detachment. No protease was required for cell detachment from the culture support. Besides demonstrating cell temperature-induced cell detachment, our results indicate that the present approach is well tolerated by the cells. Neither the polymer nor the detachment method reduced cellular metabolic activity. This promising approach is a new alternative for cells sensitive to the proteolytic treatment routinely used for cell detachment. The presented method may be extended to other cells as the RGD sequence is widely recognized. Besides, the chemical route indicated herein can be applied to different peptide sequences, which may be advantageous in order to match specific adhesion requirements of other cell types. An additional convenience of our approach relates to the film deposition technique. Solvent casting allows straightforward cost-effective film formation also enabling deposition on a wide range of substrates, such as broadly available tissue culture plastic or glass of different sizes. This is a quite desirable requisite[18] conferring a high plasticity on our approach.

AUTHOR INFORMATION

Corresponding Author

*E-mail: omerten@genethon.fr.

Present Address

[‖]Laboratoire Matière et Systèmes Complexes, UMR 7057, CNRS and Université Paris Diderot, 10 rue Alice Domon et Léonie Duquet, 75205 Paris cedex 13, France.

Notes

The authors declare no competing financial interest.

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