Acta Biomaterialia 59 (2017) 117–128

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Thermoresponsive poly(glycidyl ether) brushes on gold: Surface engineering parameters and their implication for cell sheet fabrication

Silke Heinen, José Luis Cuéllar-Camacho, Marie Weinhart *

Institute of Chemistry and Biochemistry, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 29 March 2017
Received in revised form 28 May 2017
Accepted 20 June 2017
Available online 21 June 2017

Keywords:
Cell sheet fabrication
Grafting density
Molecular weight
Thermoresponsive
Self-assembled monolayer
Brushes
Protein adsorption
AFM
SPR

ABSTRACT

Thermoresponsive polymer coatings, optimized for cell adhesion and thermally-triggered cell detachment, allow the fabrication of confluent cell sheets with intact extracellular matrix. However, rational design guidelines for such coatings are rare, since temperature-triggered cell adhesion and detachment from thermoresponsive surfaces are mechanistically not well understood. Herein, we investigated the impact of molecular weight (2, 9, 24 kDa), grafting density (0.04–1.4 chains nm^{-2}), morphology, and roughness of well-characterized thermoresponsive poly(glycidyl ether) brushes on the cell response at 37 and 20 °C. NIH 3T3 mouse fibroblasts served as a model cell line for adhesion, proliferation, and cell sheet detachment. The cell response was correlated with serum protein adsorption from cell culture medium containing 10% fetal bovine serum. Intact cell sheets could be harvested from all the studied poly(glycidyl ether) coated surfaces, irrespective of the molecular weight, provided that the morphology of the coating was homogenous and the surface was fully shielded by the hydrated brush. The degree of chain overlap was estimated by the ratio of twice the polymer's Flory radius in a theta solvent to its interchain distance, which should be located in the strongly overlapping brush regime ($2\ R_f/l > 1.4$). In contrast, dense PNIPAM (2.5 kDa) control monolayers did not induce protein adsorption from cell culture medium at 37 °C and, as a result, did not allow a significant cell adhesion. These structural design parameters of functional poly(glycidyl ether) coatings on gold will contribute to future engineering of these thermoresponsive coatings on more common, cell culture relevant substrates.

Statement of Significance

Cell sheet engineering as a scaffold-free approach towards tissue engineering resembles a milestone in regenerative medicine. The fabrication of confluent cell sheets maintains the extracellular matrix of cells which serves as the physiological cell scaffold. Thermoresponsive poly(glycidyl ether)s are highly cell-compatible and brushes thereof promote cell adhesion and growth without modification with additional cell adhesive ligands. Thus, a direct correlation of temperature-dependent serum protein adsorption and cell response with surface design parameters such as grafting density and molecular weight became accessible. Hence, surface engineering parameters of well-defined poly(glycidyl ether) monolayers for reproducible cell sheet fabrication have been identified. These design guidelines may also prove beneficial in the development of other brush-like thermoresponsive coatings for cell sheet engineering.

1. Introduction

Thermoresponsive polymers with a lower critical solution temperature (LCST) between 25 and 35 °C, such as poly(*N*-isopropyl acryl amide) (PNIPAM), are promising candidates for a functional coating material of *in vitro* cell culture substrates [1,2]. The general concept was introduced by Okano et al. in 1990 with PNIPAM-grafted polystyrene dishes, which were produced by electron-beam polymerization [3]. Ideally, the thermoresponsive polymer-grafted surfaces allow regular cell adhesion and proliferation under standard cell culture conditions at 37 °C. At 20 °C, the thermoresponsive coatings promote an enzyme-free, solely temperature-triggered, mild cell detachment, and harvest of adherent cells from the surface [4]. As a result, not only singularized cells with intact glycocalix but also confluent cell sheets together with their extracellular matrix (ECM) become accessible for tissue engineering and

* Corresponding author.
E-mail addresses: silke.heinen@fu-berlin.de (S. Heinen), luis.cuellarcamacho@fu-berlin.de (J.L. Cuéllar-Camacho), marie.weinhart@fu-berlin.de (M. Weinhart).

http://dx.doi.org/10.1016/j.actbio.2017.06.029
1742-7061/

regenerative medicine. On many thermoresponsive coatings, however, cell adhesion is not supported. Thus, an additional modification with cell adhesion-promoting molecules such as RGD peptides or fibronectin is required [5,6]. In search of novel, PNIPAM-free, cytocompatible alternatives for cell sheet engineering with improved cell adhesion properties, thermoresponsive monolayers of poly(2-alkyl-2-oxazoline) [7,8], poly[tri(ethylene glycol) monoethyl ether methacrylate] [9,10], poly(*N*-vinylcaprolactam) [11], and poly(glycidyl ether)s [12,13], have proven their applicability in cell sheet fabrication. Focusing on structurally well-defined monolayer coatings, meaningful correlations of surface parameters and biological response are facilitated. For poly(glycidyl ether)s a proof of concept study was reported with a 2 kDa random copolymer of glycidyl methyl ether (GME) and glycidyl ethyl ether (EGE) assembled as a monolayer coating on gold [13]. The cell response of NIH 3T3 mouse fibroblasts has been correlated to the comonomer ratio of the polymer tethered to the surface [12]. Polymers of two comonomer ratios (1:3 and 1:5 GME/EGE, respectively) have been identified to allow cell sheet fabrication. Faster cell adhesion and proliferation of NIH 3T3 cells was found on polymer coatings with a 1:3 GME/EGE comonomer ratio.

However, rational surface design guidelines for the fabrication of functional, thermoresponsive coatings with optimized cell response are generally lacking. Although in-depth studies on thermoresponsive brushes have been performed either with respect to physical surface parameters [14–17] or cell interactions [18,19], correlation of both are rarely found in the literature. In this regard, Dworak et al. reported on poly(2-alkyl-2-oxazoline) brushes, which were prepared by a combined living polymerization/termination reaction on an amino-functionalized glass surface [8]. Human dermal fibroblasts adhered on those poly(oxazoline) brushes (14, 20, 42 kDa) of varying chain grafting density (0.16–0.26 chains nm^{-2}) similarly as observed with the tissue culture polystyrene (TCPS) control. The cell sheets detached at 20 °C within 30 min from these surfaces [8]. Furthermore, PNIPAM brushes with varying grafting densities of 0.02, 0.03, and 0.04 chains nm^{-2} and different molecular weights (23, 49, 58 kDa) prepared by surface-initiated RAFT polymerization were correlated with the cell response by Okano and coworkers [20]. Reproducible detachment of cell sheets of bovine carotid artery endothelial cells was only ensured, for all tested molecular weights, at a PNIPAM grafting density of 0.04 chains nm^{-2}. Thereby, minimal detachment times of 30 min were reported for the highest molecular weight PNIPAM (58 kDa). From this study, it can be extracted that, at a relatively low surface grafting density of 0.03 chains nm^{-2} a minimal PNIPAM molecular weight of 49 kDa is required for effective detachment of confluent cell sheets [20].

Herein, we correlate the temperature-dependent biological response (proteins and cells) with detailed structural features of thermoresponsive monolayer coatings of poly(GME-*ran*-EGE) copolymers. As a result, comprehensive surface design guidelines for poly(glycidyl ether)-coated surfaces with application in cell sheet fabrication have been derived in terms of grafting density, molecular weight of the polymer, morphology, and roughness of the coating. Furthermore, the respective Flory radius R_f of the polymers in a theta and in a bad solvent was calculated to approximate the radius of the surface-tethered polymer chain under cell detachment (20 °C) and cell seeding (37 °C) conditions. The individual degree of chain overlap (2 R_f/l) within the different coatings at low and high temperatures was estimated from twice the Flory radius in relation to the anchor distance l of the polymers on the surface. In doing so, a fair and meaningful comparison among thermoresponsive brush-like polymer coatings and their performance in cell sheet fabrication became possible.

2. Materials and methods

2.1. Materials

Gold-coated substrates, such as SPR-Au-sensors, semitransparent gold-coated glass for cell culture and microscopy, and (1 1 1) gold-glimmer films on mica, were obtained from GE Healthcare Bioscience AB (Uppsala, Sweden), Ssens (Enschede, Netherlands), and Georg Albert PVD (Silz, Germany), respectively. Thermoresponsive thiol/disulfide-functionalized poly$(GME)_n$-*ran*-$(EGE)_{3n}$ **1** ($C_{11}SH$; 2 kDa), **2** (C_8SS-; 2 kDa), **3** (C_4SH; 2 kDa), **4** (C_4SH; 9 kDa), **5** (C_4SH; 24 kDa), and PNIPAM **6** (C_4SH; 2.5 kDa) were synthesized as published previously [21,22]. All other chemicals were purchased from Sigma Aldrich (Steinheim, Germany). Deionized water (MQ) was generated by a Millipore water purification system with a minimum resistivity of 18.0 MΩ cm. For SPR measurements phosphate buffered saline (PBS, pH 7.4) without calcium and magnesium ions reconstituted from PBS tablets (P4417-100TAB, Sigma Aldrich) was used.

2.2. Gold surface modification via monolayer formation

Prior to polymeric monolayer formation, gold-coated glass substrates (SPR sensors and semitransparent gold slides) were treated with Piranha solution (H_2SO_4:H_2O_2 = 3:1) for 20 s. The substrates were subsequently rinsed with MQ water and ethanol and dried in a nitrogen gas stream. (Caution: Piranha is an aggressive and explosive chemical; check the safety precautions before using it.) Au (1 1 1) on mica was used as received for monolayer formation and subsequent atomic force microscopy (AFM) studies. Monolayers were formed on gold-coated substrates from thermoresponsive poly$(GME)_n$-*ran*-$(EGE)_{3n}$ (2–3, 9, 24 kDa) or PNIPAM (2.5 kDa), equipped with a thiol or disulfide anchor group as published previously [22]. In brief, cleaned gold substrates were immersed in 0.5 mM polymer solutions in ethanol or PBS for three hours at 20 or 32 °C, respectively. At 32 °C all polymer solutions in PBS appeared turbid and hence the polymer chains at this temperature were in a collapsed state. Grafting under these conditions led to the formation of polymer aggregates, which loosely adsorbed on the surface. In order to guarantee high reproducibility when coatings were prepared at 32 °C in aqueous solution, three interim additional washing steps with ethanol were incorporated into the coating procedure to remove aggregate structures [22]. This specific coating protocol at 32 °C is herein called cloud point grafting (CPG). Varying reproducible grafting densities from 0.04 to 1.41 chains nm^{-2} were adjustable depending on the anchor group used, molecular weight, and the grafting conditions (CPG vs grafting from ethanol). Apparent grafting densities were deduced from "wet" and "dry" layer thicknesses obtained by QCM-D and ellipsometry measurement and were calculated as described previously [22]. In brief, "wet" and "dry" areal masses were calculated from the measured layer thickness multiplied by the approximated layer density ($\delta_{Layer\text{-}wet}$ = 1.0 g cm^{-3}, $\delta_{Polymer\text{-}dry}$ = 1.2 g cm^{-3}) in order to deduce the percentage of ethanol in the solvated layer and subsequently the "effective, solvated" molecular weight of the polymers within the respective coatings. Furthermore, the grafting densities of the polymer chains within the solvated polymer layers were calculated from the "wet" areal mass of the coating and the Avogadro constant divided by the "solvated" molecular weight. Sessile drop water contact angles of all investigated coatings were in the range of 50°–72° at 20 °C [22]. In addition, contact angles on p(GME-*ran*-EGE) coatings were determined below and above the expected transition temperature of the polymers by three different methods, the sessile drop technique with a water drop in air or a water drop in decane, as well as a captive air bubble in water. No significant