Biomaterials 102 (2016) 268–276

Contents lists available at ScienceDirect

Biomaterials

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

N-terminal specific conjugation of extracellular matrix proteins to 2-pyridinecarboxaldehyde functionalized polyacrylamide hydrogels

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ARTICLE INFO

Article history:
Received 5 March 2016
Received in revised form
8 June 2016
Accepted 9 June 2016
Available online 15 June 2016

ABSTRACT

Polyacrylamide hydrogels have been used extensively to study cell responses to the mechanical and biochemical properties of extracellular matrix substrates. A key step in fabricating these substrates is the conjugation of cell adhesion proteins to the polyacrylamide surfaces, which typically involves nonspecifically anchoring these proteins via side-chain functional groups. This can result in a loss of presentation control and altered bioactivity. Here, we describe a new functionalization strategy in which we anchor full-length extracellular matrix proteins to polyacrylamide substrates using 2-pyridinecarboxaldehyde, which can be co-polymerized into polyacrylamide gels and used to immobilize proteins by their N-termini. This one-step reaction proceeds under mild aqueous conditions and does not require additional reagents. We demonstrate that these substrates can readily conjugate to various extracellular matrix proteins, as well as promote cell adhesion and spreading. Notably, this chemistry supports the assembly and cellular remodeling of large collagen fibers, which is not observed using conventional side-chain amine-conjugation chemistry.

1. Introduction

Cells are sensitive to the mechanical properties of their environment. In particular, the stiffness of the extracellular matrix (ECM) substrate to which cells adhere can affect cell morphology, adhesion, migration and stem cell differentiation [1–6]. To study this behavior in culture, polyacrylamide (PAAm) hydrogels are most commonly used because the compliance of this material can easily be tuned to mimic stiffness values of soft tissues [2–4,7–12]. Additionally, PAAm does not promote protein adsorption or cell adhesion, enabling improved control over substrate functionalization with cell-adhesive ligands [8,10,11].

The functionalization of PAAm with the ECM proteins required for cell adhesion has been achieved using pendant *N*-hydroxysuccinimide (NHS) esters that acylate lysine residues [13], hydrazides that couple to periodate-oxidized glycans on proteins [14], non-covalent adsorption [15], and biotin–streptavidin interactions [16]. Perhaps the most common strategy uses a heterobifunctional crosslinker, sulfo-SANPAH (**1**, Fig. 1a–c), which inserts into the PAAm backbone following photolysis of the aryl azide moiety [7,8]. The resulting polymer-linked NHS ester is then used to acylate amine groups on proteins. Although widely used, this process can create significant batch-to-batch variability, and the immobilized NHS esters are subject to competitive hydrolysis under protein attachment conditions. Moreover, NHS esters can react with any amines on the proteins, resulting in nonspecific tethering at multiple sites with uncontrollable and unpredictable adhesive ligand presentation (Fig. 1b). The orientation of immobilized ligand has been shown to influence the accessibility of epitopes and affect cell behavior [17]. Furthermore, protein structure and activity may be affected if functionally and structurally important lysine residues are engaged with the surface.

This conjugation approach has also contributed to a major controversy in the field, questioning whether the density of these

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http://dx.doi.org/10.1016/j.biomaterials.2016.06.022
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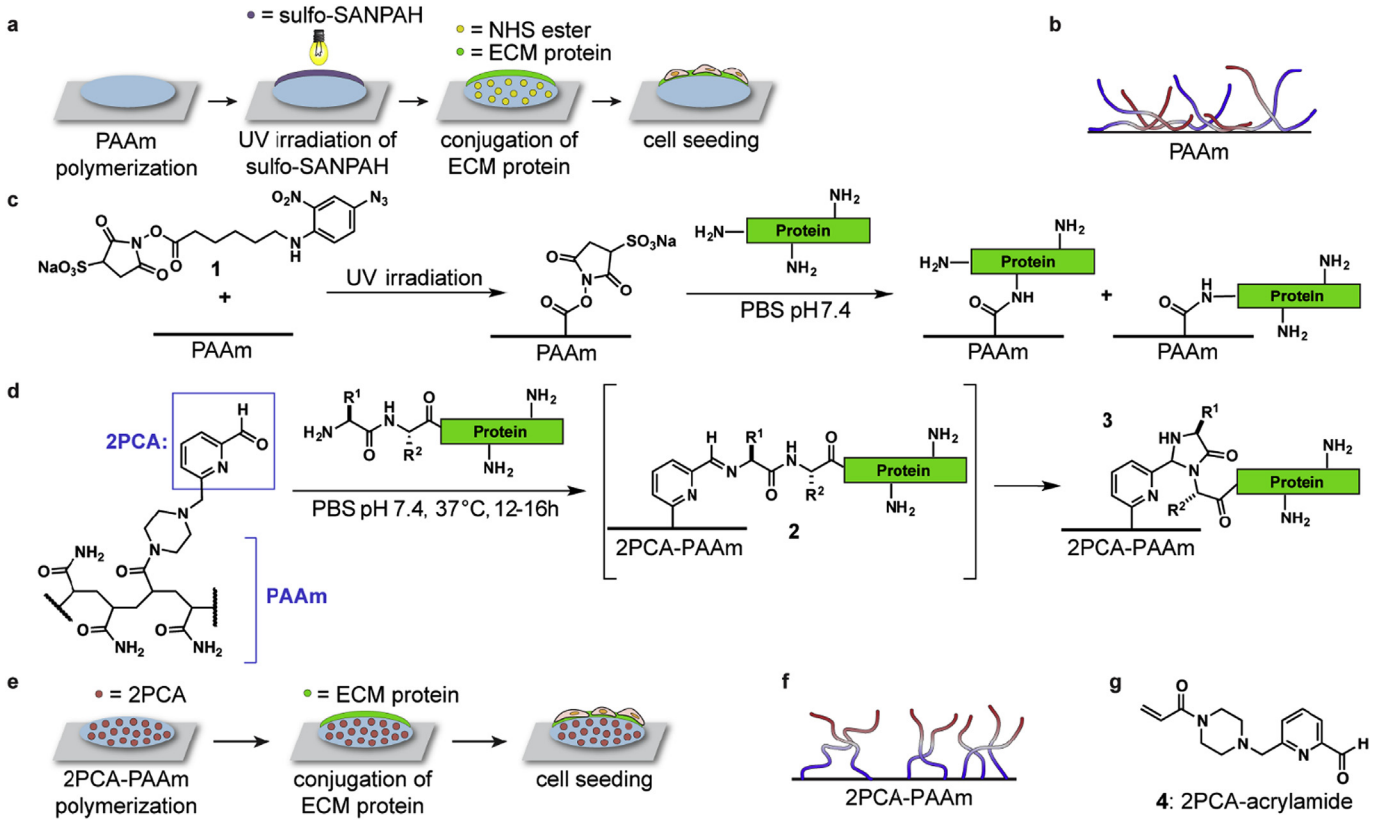

Fig. 1. ECM protein conjugation on polyacrylamide (PAAm) substrates. (a–c) A scheme is shown for ECM protein conjugation using sulfo-SANPAH (**1**). This reaction targets all available amines, resulting in heterogeneous protein attachment. (d–f) In this work, protein N-termini are coupled to 2-pyridinecarboxaldehyde (2PCA)-modified PAAm gels, resulting in a more uniform presentation. (g) A 2PCA derivative for acrylamide copolymerization is shown. Protein N-termini are shown in blue in (b) and (f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lateral tethers directly affects cell behavior and function [18–20]. It has been hypothesized that due to smaller pore size, stiffer substrates have shorter distances between surface anchorage points. This reduces the local deformability of these proteins relative to softer substrates, which have comparatively greater surface porosity and longer tethering distances, implying that local tether density controls cell behavior [18]. In contrast, a subsequent study showed that systematically varying PAAm porosity without altering stiffness does not significantly influence protein tethering, substrate deformation, or stem cell differentiation, implying that cells respond to bulk substrate stiffness rather than the degree of protein tethering [19]. Nevertheless, anchoring density was still observed to increase with increasing sulfo-SANPAH crosslinker concentration, complicating data interpretation [19]. Such studies have stimulated strong interest to develop ECM-hydrogel conjugation strategies with predictable and well-controlled attachment chemistry and ligand presentation.

This problem can be addressed by developing immobilization strategies that only anchor the ECM proteins to the hydrogel surface once, thereby ensuring that each protein is tethered predictably and with minimal perturbation of protein structure and function. Site-selective protein immobilization strategies using native cysteine residues, small molecule probes, or peptide fusion tags have been developed for substrates other than PAAm, but they are generally incompatible with commercially-available tissue-purified ECM proteins [21–23]. As a result, there remains a significant need for site-specific strategies to immobilize ECM proteins to hydrogel surfaces for cell culture applications.

We report herein a well-defined immobilization strategy in which native proteins are conjugated to PAAm hydrogels specifically through their N-termini using recently published 2-pyridinecarboxyaldehyde (2PCA) conjugation chemistry (Fig. 1d–f) [24]. We demonstrate that this immobilization strategy is applicable to multiple widely used ECM proteins. Cells readily adhere and spread on these substrates, and despite the presence of only a single protein-hydrogel tethering point, cells recapitulate stiffness-dependent behavior. Moreover, 2PCA-conjugated surfaces remarkably and uniquely support the assembly of attached collagen chains into large fibers, which can be remodeled and bundled by attached cells in a manner reminiscent of collagen remodeling in tissue.

2. Materials and methods

2.1. Reagents and instruments

Unless stated otherwise, all reagents and solvents used were of analytical grade and were used as received from commercial sources. Type I bovine collagen (PureCol, Advanced BioMatrix), human plasma fibronectin (Millipore) and mouse laminin (Gibco) were also used as received. PBS, pH 7.4, was purchased from Fisher Scientific.

NMR spectra were recorded on a Bruker AVQ-400 spectrometer. ^{1}H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 7.26). Multiplicities are reported as: s (singlet), br.s (broad singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets) or m (multiplet). Coupling constants are reported as a J value in Hertz (Hz). ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 77.2). High-resolution electrospray ionization