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Surface microstructuring and protein patterning using hyaluronan derivatives

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ABSTRACT

Natural polymers, such as hyaluronan, are considered as good candidates to substitute synthetic materials in many biological applications due to their intrinsic biocompatibility for *in vitro* and living cell experiments. This work describes surface modifications performed over modified hyaluronan derivatives which could help to understand the physical and biochemical cues on the cell-behaviour. A photocrosslinkable methacrylated hyaluronan was microstructured using soft lithography techniques to obtain a microenvironment suitable for cell-behaviour experiments, which might mimic the extra-cellular matrix. In addition, sulphated and non-sulphated oxidized hyaluronan were immobilized on bare substrates and micrometric features of cell adhesive and non-adhesive proteins were patterned by microcontact printing on top of them. The obtained structures were characterized by optical microscopy, profilometry and atomic force microscopy. The stability of structures was tested by immersion in physiological salt solutions. The observed results prove the suitability of the materials and protocols described.

1. Introduction

Natural polymers have been extensively used in biological research, tissue engineering, diagnostics and screening [1,2]. Among them, glycosaminoglycans (GAGs) are large complex carbohydrate molecules related to many physiological processes. These molecules are present on all animal cell surfaces in the extracellular matrix (ECM) [3] and they include two main types: non-sulphated GAGs, such as hyaluronan (HA) and sulphated GAGs (e.g. chondroitin sulphate, heparin). Since they are found throughout the body, these GAGs offer much better biocompatibility than synthetic polymers, a prerequisite for *in vitro* cell-based studies and devices. In fact, HA has been approved and used clinically [4] and it is considered a key biomaterial for the development of tissue engineering [2].

Traditionally, the interactions of GAGs with proteins and cells have been studied in solution. However, since GAGs in the ECM are immobilized (covalently or physically), *in vitro* immobilized GAGs may better mimic the *in vivo* environment for cell culture experiments. Therefore, the interaction of cells with substrates is a vast field of intense research due to its relevance for very different applications [5]. Since the ECM possesses different topographical and adhesive features, the response of cells to surface topography, such as grooves, pillars, etc. has been studied extensively [6]. The ECM also exposes adhesive and non-adhesive features which have been mimicked *in vitro* by surface-patterning of proteins [7]. However, the way the micro or nanotopography regulates cell behaviour is not well understood [8]. Due to this lack, there is a need for developing adequate functional surface patterns for *in vitro* cell research.

Topographical patterns for cell studies are considered a powerful mechanism to alter, direct and control *in vitro* cell behaviour from cell adhesion to gene expression [8–10]. Despite some promising results, these structures still do not completely mimic the microenvironment found in the ECM [7]. Since it is known that GAGs have a biochemical effect on the cells, the combination of mechanical effects by microstructuring and biochemical cues induced by GAGs is an interesting field of research. Unfortunately, the high solubility of these materials is a problem when cell-culture experiments are carried out [11]. However, by using

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photocrosslinkable HA derivatives, whose properties can be modified by adjusting the crosslinking density of the resulting hydrogel, suitable microenvironments have been already created [2,12–14].

The present work describes surface structuring on modified HA derivatives. Methacrylated hyaluronan (MAHA) has been microstructured by photocrosslinking and soft lithography techniques to obtain a microenvironment emulating the ECM. In another approach, two modified GAGs, oxidized hyaluronan and oxidized sulphated hyaluronan (ox-HA and ox-HAS), have been covalently immobilized onto bare substrates giving rise to a self-assembled monolayer. Resistant micrometric features of cell adhesive (fibronectin, FN) and non-adhesive (bovine serum albumin, BSA) proteins have been transferred to these substrates by microcontact printing (μCP) [15]. The resulting structures were characterized by optical microscopy, profilometry and atomic force microscopy (AFM) and their resistance to immersion was tested showing that they could be used for cell-behaviour studies.

2. Materials and methods

MAHA with a degree of substitution (average number of methacrylated groups per disaccharide repeating unit of HA) of 0.9 was prepared starting from native high-molecular weight HA (M_W = 923.6 kDa) according to a reported procedure [16]. Ox-HA with 10% oxidation percentage (104 kg/mol, polydispersity index of 1.66) and ox-HAS with 11.3% oxidation percentage and a sulphate degree of 2.4 were also synthesized. 3-(Trimethoxysily)propyl methacrylate (TMSPM), 11-amino-1-undecanethiol hydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1 M buffering agent, sodium cyanoborohydride ($NaBH_3CN$), triethylene glycol dimethacrylate (TEGDM), Dulbecco's phosphate buffered saline (PBS) and hexamethyldisilazane 98% (HMDS) were purchased from Sigma–Aldrich. 3-Aminopropyl trimethoxysilane (APTMS) and 1H, 1H, 2H, 2H-perfluorooctyl trichlorosilane (FOTS) were obtained from ABCR GmbH. A Sylgard 184 poly(dimethyl siloxane) (PDMS) was purchased from Dow Corning. Irgacure 369 was obtained from CIBA. Alexa Fluor 488-conjugated BSA (BSA Alexa Fluor 488), fibronectin (FN) were provided by Fisher Scientific SL. Hilyte 488-conjugated fibronectin (FN 488) was purchased from Cytoskeleton. Ultrapure water was used for all dilutions and washings and all reagents were of analytical grade.

2.1. Fabrication of silicon masters

Silicon masters, for PDMS stamps fabrication, with microstructures consisting of grooves from 4 to 200 μm of periodicity were fabricated by UV photolithography and deep reactive ion etching (DRIE). Four inch silicon wafers were first dehydrated at 180 °C for 30 min in a convection oven. A first layer of an adhesion promoter, HMDS, and a positive photoresist (maP-1205) were spin coated and processed using a conventional UV-Lithography process. These patterns were transferred to the silicon by DRIE (Oxford Plasmalab System 80) using a combined SF_6/C_4F_8 plasma. Silicon stamps with two different depths (240 nm for micro-structuration studies and 5 μm for μCP experiments) were obtained depending on the etching time. Finally, FOTS was evaporated over the silicon stamp in a vacuum desiccator during 30 min as anti-adhesive to facilitate the PDMS stamp peeling off.

2.2. Fabrication of PDMS stamps

PDMS stamps were fabricated using the silicon masters described in the previous section. To cure the PDMS prepolymer, a mixture of 10:1 silicone elastomer and the curing agent was cast over silicon masters and placed at 60 °C overnight. PDMS was then carefully peeled off from the silicon master and cut into 10 mm × 10 mm stamps. The protruding ridges in the masters resulted in PDMS replicas with grooves. Finally, the stamps were cleaned with 70% ethanol, sonicated for 5 min and let dry prior to use.

2.3. Substrate preparation

2.3.1. Glass substrates

Four inch glass wafers were diced into 10 mm × 10 mm substrates and cleaned prior to use by copious rinsing with acetone, isopropyl alcohol and ultrapure water.

Glass substrates were methacrylated after 5 min O_2 plasma treatment by deposition of a drop of TMSPM. The substrates were kept in an oven at 100 °C for 1 h followed by 15 min at 110 °C [2]. Substrates were rinsed with ultrapure water and dried under a N_2 flow.

Amination of glass substrates for subsequent ox-HA and ox-HAS deposition was performed by evaporation of APTMS.

2.3.2. Gold substrates

Another set of glass wafers was cleaned as described in Section 2.3.1. Thereafter, a 40 nm thick gold layer was deposited onto the wafers by electron beam physical vapour deposition (ATC Orion series UHV Evaporation system, AJA International Inc.). A previous evaporation step of a 3 nm thick layer of titanium was carried out to improve adhesion to the glass. These wafers were also diced into 10 mm × 10 mm substrates.

These substrates were cleaned using a piranha solution (H_2SO_4:H_2O_2, 3:1) for 10 min, rinsed with ethanol and dried using N_2. Subsequently, substrates were immersed in a 20 μM solution of 11-amino-1-undecanethiol hydrochloride in pure ethanol for 48 h. After amination, substrates were washed with ethanol and dried under a N_2 flow to be ready for GAG immobilization.

2.4. Microstructuring of MAHA

Two percent and 5% w/v MAHA solutions in water were filtered using 0.2 μm diameter porous filters (Millex, Millipore) and blended with 0.3% m/v Irgacure 369 in *N*-vinyl pyrrolidone and with TEGDM as an additional crosslinker using a MAHA:TEGDM 1:1 ratio. One hundred and thirty microlitres of these mixtures were deposited onto the microstructured PDMS stamps and the methacrylated glass substrates were carefully placed over them. Photopolymerization was performed under UV light from a 350 W short arc lamp (Advanced Radiation Corporation, 350–450 nm, 10 mW/cm^2) for 2 h under a N_2 atmosphere. After UV exposure, the PDMS stamp was left overnight before demoulding to ensure the stabilization of the hydrogel microstructures (Fig. 1a). After demoulding of the stamps from the microstructured MAHA, a Veeco Dektak 8 (Veeco Instruments, Plainview, NY) mechanical profilometer was used for profile measurements.

2.5. Immobilization of ox-HA and ox-HAS GAGs over aminated gold/glass substrates

Ox-HA and ox-HAS were immobilized onto substrates as follows: each well of a 12-well plate containing a single aminated gold or glass substrate was filled with 1 ml of 4 mg/ml ox-HA or ox-HAS solution in PBS. The plates were kept shaking gently for 24 h. One millilitre of $NaBH_3CN$ (3 mg/ml in PBS) was added into each well and kept at 4 °C for another 24 h. After the ox-GAG immobilization, the coated surface was once more rinsed with water and dried under a N_2 stream.