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A novel multistep method for chondroitin sulphate immobilization and its interaction with fibroblast cells

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ABSTRACT

Polymeric biomaterials are widely used in medical applications owing to their low cost, processability and sufficient toughness. Surface modification by creating a thin film of bioactive agents is promising technique to enhance cellular interactions, regulate the protein adsorption and/or avoid bacterial infections. Polyethylene is one of the most used polymeric biomaterial but its hydrophobic nature impedes its further chemical modifications. Plasma treatment is unique method to increase its hydrophilicity by incorporating hydrophilic oxidative functional groups and tailoring the surface by physical etching. Furthermore, grafting of polymer brushes of amine group containing monomers onto the functionalized surface lead to strongly immobilized bioactive agents at the final step. Chondroitin sulphate is natural polysaccharide mainly found in connective cartilage tissue which used as a bioactive agent to immobilize onto polyethylene surface by multistep method in this study.

1. Introduction

Polymeric materials have been widely used for various industrial applications such as packaging, automotive and aeronautics due to their low cost, sufficient mechanical properties, chemical stability and processability. In the biomedical area (i.e. implants, wound dressing, suture, etc.) some of the natural and synthetic polymers are also widely used besides metals, ceramics and composites as a polymeric biomaterials. Thereby, except their mechanical and chemical stability, surface properties play an important role owing to direct contact with the living tissue when placed into the body. Principally, non-toxicity is one of the required features for prospective implants. Moreover, infections stemming from microbial interactions (especially, nosocomial infections during hospitalization) have to be taken into account [1–3]. Thus, insufficient cellular interactions in terms of cell adhesion and proliferation which is known as a disadvantage of the polymeric biomaterials need to be promoted. Surface modification of polymers is beneficial for changing the surface characteristics in order to minimize the microbial attachment to avoid infections and maximize the cell adhesion and proliferation ability to accelerate the tissue regeneration. Surface modification changes polymer's surface energy, polarity, topology and chemical composition. Therefore, changes its interaction properties with the environment, without affecting its bulk properties since interaction is limited by the surface top layer [4,5]. Furthermore, surface coating of polymeric biomaterials by using chemical agents (both synthetic and natural materials) is promising approach for enhanced surface interactions with the living tissue according to agent's specific properties. There are several methods to modify a polymer surface: chemical vapour deposition (CVD), wet-chemistry, ozone induced treatment, corona discharge, UV irradiation, flame treatment and plasma treatment [6–9]. It is necessary to take into account the heat range during the treatment process to keep the advantageous of polymer's bulk properties and toxic residues content minimization after the treatment. Plasma treatment is a fast and promising process to modify the polymer surface in relatively short time without exposing heat and chemicals. Modified surface characteristics by plasma depends primarily on plasma parameters, such as applied discharge gas and its flow rate, processing pressure, plasma reactor type, applied power, frequency and exposure time. By moderating those parameters specific type of plasma can be obtained for individual applications such as ion etching, deposition, polymerization, cross-linking, chain scission, surface activation, sterilization, etc. [8,10,11]. Plasma contains metastables, ions, electrons, neutral species and photons which interact with the polymer surface in order to increase its surface energy, hydrophilicity and modify the surface chemistry via incorporation of oxygen containing functional groups such as hydroxyl, carboxyl, carbonyl, peroxide in the case of using air as a carrier plasma [8,12–14]. Incorporation such functional groups makes polymer more capable to interact with further reagents in chemical processes to obtain special properties for specific applications by means of coatings by various biomolecules responsible for desired surface properties.

Besides limited surface interactions with the living tissue, it has been demonstrated that some of the polymers (i.e., polyurethane, polyethylene terephthalate, polyglycolic acid and polylactic acid copolymers, polycaprolactone, etc.) coated on drug-eluting stents caused long term inflammation [15]. Thus, research interest shifted to polysaccharides

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due to their excellent biocompatibility, superior cellular adhesion and proliferation [15,16]. Chondroitin sulphate (ChS) is one of the most important natural polysaccharide mainly found in connective cartilage tissue [15,17] and also in other sources as a extracellular matrix [16] which has an important role on cell functions [18,19]. It is a linear, polydisperse, sulphated polysaccharide which belongs to glycosaminoglycans (GAGs) family [20–22]. It has highly negative polarity due to SO_4^{-2} and COO^- presence [19] which plays significant role on interaction with other constituents by means of repulsive and attractive forces. ChS has very complex heterogeneous structure [20] and occurs in several forms, i.e., Chondroitin 4-sulfate (ChS A), Chondroitin 6-Sulfate (ChS C), dermatan sulphate (ChS B) [17]. ChS is generally produced by extraction and purification from animal tissues [20,23]. ChS has beneficial properties for tissue engineering such as anti-inflammatory effect, wound healing capability and ability to accelerate the regeneration of injured bone [16,18]. It is also used as a dietary supplement for the osteoarthritis treatment [20,23]. Due to the fact that effect of the orally delivered agent is reduced by the digestive system, ChS immobilization on the selected biomaterials (named as surface mediated drug delivery) will have higher concentration, thus, increased effect on the particularly contacted tissue in surgical applications [17].

Modified LDPE surface by plasma introduces negatively charged functionalities to the surface and further ChS with negative polarity immobilization create electrostatic repulsive force which reduces the binding affinity between them. To avoid this, positively charged mediators, like allylamine (CH_2═CH—CH_2—NH_2) are promising choice to introduce a high density of positively charged amine groups (—NH_2) by grafting with a good stability [24–26].

In the clinical tissue engineering fibroblast cells, found in connective tissues, are responsible for wound healing and regeneration of the injured tissue [27,28]. Therefore, cell interaction with the modified biomaterial surface (i.e., adhesion, proliferation and differentiation) play a key role for the regeneration of the injured tissue in the living body.

In this study, LDPE as one of the most common polymeric material in medical applications area has been used as a substrate. RF plasma discharge has been applied with air as a carrier gas onto LDPE surface to create functional groups which are able to react with selected reagents mentioned below, as initiators of co-polymerization in order to obtain satisfactory adhesion interaction properties. Allylamine (AAM), *N*-allylmethylamine (MAAM) and *N*,*N*-dimethylallylamine (DAAM) have been selected as monomers to graft onto treated LDPE surface by co-polymerization process to create polymer brush as a positively charged mediator by means of exposure to monomer vapours. Samples were consequently taken out of the vapours and immersed to ChS solution to immobilize it onto the surface. The effect of plasma treatment on morphology of LDPE and surface conditions of coated samples has been investigated by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Changes in surface energy and wettability of samples were evaluated by water sessile drop contact angle method. Chemical composition of the samples was revealed by Fourier Transform Infrared (FTIR) spectroscopy. Cell behaviour of mouse primary fibroblast was observed by fluorescence microscopy to confirm the application potential of prepared surfaces.

2. Experimental

2.1. Materials

Low density Polyethylene (LDPE) film of the 100 μm thickness was obtained and used as received without further purification in the form of square sheets (50 × 50 mm), hereafter referred to as PE. Monomers of Allylamine (AAM), *N*-allylmethylamine (MAAM), and chondroitin sulphate from bovine trachea (ChS) were obtained from Sigma Aldrich (USA) and *N*,*N*-dimethylallylamine (DAAM) was supplied by Fluka (USA). ChS solution has been prepared by dissolving 1% (w/v) of ChS in distilled water.

2.2. Plasma surface modification and reagent immobilization

Radio-frequency (RF) plasma was generated by using PICO plasma reactor (Diener, Germany) performed at 13.56 MHz frequency with 50 W of discharge matching power with air as a discharge gas with 20 standard cubic centimetre per minute (sccm) flow rate under 50 Pa chamber pressure. Both sides of each PE samples were exposed to plasma for 60 s to create free radicals and reactive species on the surface to act as initiator for further copolymerization process and hereafter referred to as PERF.

Subsequently, treated PE samples were taken out of the reactor and immediately placed to chamber containing saturated vapours of AAM, MAAM and DAAM for 10 s in order to immobilize the monomers by radical graft copolymerization process to create functional amine groups containing polymer brushes onto the surface by means of reaction with pre-formed free radicals and samples hereafter referred to as PERFA, PERFM and PERFD, respectively.

Each PERFA, PERFM and PERFD sample has been separately placed into ChS solution containing vial for 24 h at room temperature to immobilize the ChS to polymer brush of AAM, MAAM and DAAM by means of intramolecular forces. After 24 h of reaction time samples were taken out of the vials and gently dipped into water and then distilled water for cleaning of non-interacted ChS species. Finally, cleaned samples were dried for 2 h at room temperature. Such prepared samples are labelled as PERFAC, PERFMC and PERFDC according to previously created polymer brushes.

2.3. X-ray Photoelectron Spectroscopy (XPS)

Change in chemical composition was detected using a TFA (Physical Electronics, USA) X-ray photoelectron spectroscope (XPS) with a MultiPak software to analyze elemental concentration. X-rays generated with monochromatic Al $K_{\alpha 1,2}$ radiation at 1486.6 eV, under 6×10^{-8} Pa chamber pressure and exposed to samples with a 400 μm spot size. The emitted photoelectrons were detected with a hemispherical analyzer placed at angle of 45° in order to correlate to the normal plane of the samples.

2.4. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Surface chemistry examination to obtain changes in chemical compositions of the samples Nicolet iS5 (Thermo Scientific, USA) single beam Fourier transform infrared spectroscope (FTIR) equipped with iD5 attenuated total reflectance (ATR) was used. Collected spectra recorded between 400 and 4000 cm^{-1} wavelength with a resolution of 2 cm^{-1} for 64 scans using a ZnSe crystal which was placed to an incident angle of 45°.

2.5. Surface wettability evaluation

Sessile drop method was used to evaluate surface wettability of the samples via SEE System (Advex Instruments, Czech Republic) equipped with a CCD camera. Distilled water was used as a testing liquid at 22 °C and 60% relative humidity. Droplet volume of 5 μL was set for each experiment and 10 separated drops were placed to the each sample surface for 30 s to obtain average contact angle value (Q_w). Static contact angle snapshots were taken 20 s after placing the water sessile drop in all experiments.

2.6. Atomic force microscopy (AFM) investigations

For sample surface topology characteristics, Dimension Icon (Bruker, Germany) atomic force microscope (AFM) was used with a ScanAsyst-Air Si/Nitride probe (k: 0.4 N/m, Bruker, USA) with peak force tapping mode. Each sample was scanned for 5 × 5 μm area with 1 Hz frequency. Average surface roughness values (R_a) were obtained by NanoScope Analysis software.