Immobilization of a biologically active coating on a hydrophobic L-lactide-ε-caprolactone copolymer

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The electron beam radiation induced grafting method was used to attach a reactive polyacrylamide (PAA) layer (20 wt %) on the surface of a biodegradable poly-L-lactide-co-ε-caprolactone (PLLA-co-CL). The biocompatibility of graft–polymer obtained was studied by cytotoxicity test and no signs of toxicity were observed. Heparin and sol–gel-produced silica-gel coatings were successfully attached on the top of the polymeric material produced. The amount of heparin immobilized directly on the surface can be controlled by reaction conditions: reaction time, temperature and pH of the incubation solution. By using acidic conditions, up to 98 μg cm^{-2} of heparin was immobilized on the surface. The sol–gel-produced silica-gel layer formed by dipping technique was 30 μm thick and the cracking of the layer was minimal after bending several times to 90°.

1. Introduction

The use of biomaterials in clinical applications has increased dramatically during the past few decades. These materials are divided into two categories, either inert or biodegradable, according to their chemical behavior in a biological environment. The biodegradable materials provide a good alternative to conventional inert materials since they are gradually degraded in the body and allow at least a partial regeneration of tissues to take place. The majority of biodegradable materials today are organic polymers such as polylactide (PLA) or polyglycolide (PGA). In addition to synthetic organic polymers, inorganic silica-based materials, such as bioactive glasses and sol–gel-processed silica gels, are also used to construct medical devices [1]. Both materials, organic and inorganic, have numerous applications [2].

Although PLLA may have physical properties that in some respects meet or even exceed those of natural tissues, the degradation of the materials often causes adverse physiological reactions, such as tissue necrosis and inflammation due to low pH. It would clearly be an advantage if the hydrophobic PLLA could be coated with another, more biocompatible material, which would also allow the attachment of biologically active agents such as proteins, drugs and growth factors on the surface of the devices designed. Most of the biocompatible materials and the biologically active molecules are hydrophilic. Consistently, silica gel is highly hydrophilic, it is biocompatible [3, 4] and a variety of biologically active molecules can easily be composed in its structure [5]. The technology to produce silica gels is simple and versatile [6]. Unfortunately, the differences in the surface characteristics and wettability between hydrophilic and hydrophobic materials cause methodological problems for attempts to attach these two types of materials to one another.

Radiation-induced grafting of different polymers has been known for many years and has been used for tailoring material with different properties. The method allows modification of polymers in the solid state, which means that customized articles can be modified and can be utilized for polymers which are biodegradable, such as PLA [7] and polycaprolactone (PCL) [8], or non-degradable, like polyethylene (PE) [9]. Examples of applications are polymer-supported catalysts [10], polymer surfaces with mucoadhesion [11] as well as materials for immobilization of biomolecules [12].

Heparin is a strongly acidic mucosaccharide that has many functions [13]. One of them is to act as a blood anticoagulant. Heparin binds antithrombin III (ATIII) to form a complex that deactivates all the serine proteases that are responsible for the coagulation cascade. The active site responsible for binding to ATIII is pentasaccaride segment (Fig. 1) [14, 15].

In this article, we describe the use of electron beam radiation for chemical modification of polymer for improving the attachment of heparin and silica gel on the surface of the polymers. Heparin and sol–gel-processed silica gel are excellent examples of biologically active surfaces, since they have many functions in biological applications.

2. Experimental procedure

The surface properties of the copolymers of PLLA (Neste Ltd) and PCL (Sigma Co.) (PLLA/PCL = 50/50) were altered by grafting with acrylamide (Promega Co.) onto the surface of PLLA-co-CL by electron beam (EB) irradiation [16]. The grafted polymer sheets were washed thoroughly with ethanol and deionized water. The grafted surface was then able to attach silica gel or biologically active molecules such as heparin.

The grafted polymer was allowed to react with heparin solution (0.025 mg heparin/5 ml incubation solution). Sodium salt of heparin was obtained from Orion Pharma (biological activity 139 IU mg^{-1}). pH of the solution was adjusted before heparin addition, by acetic acid buffer (pH 4.5) and TRIS or phosphate buffer (pH 8). The polymer sheets were incubated with this heparin solution. The incubation times were varied from 2 to 96 h and incubations were carried out at 25 or 37 °C. Sheets with attached heparin were washed thoroughly with deionized water.

The silica sol immobilized heparin was prepared by the two-step sol–gel process using nitric acid as a catalyst [17]. The following reagents were used: tetraethoxysilane (TEOS) (Aldrich), deionized water, nitric acid (HNO_3) (Merck), ammonium hydroxide (NH_4OH) and heparin sodium salt (Orion, biological activity 139 IU mg^{-1}). To obtain 100 ml hydrolysis solution 48 g of TEOS, 45 g of deionized water and 10.1 g of catalyst (0.04 M HNO_3) were added to a glass beaker and stirred until the ingredients formed a homogeneous solution. The silica gel coating was applied by dipping grafted polymer sheets into homogeneous hydrolysis solution.

The following functional tests were performed: the biocompatibility of the materials was examined by using cultured cells [18], the release of heparin and silica were studied by dissolution test [19] and biological activity of the bound and released heparin was determined by the thrombin assay [20]. From the dissolution test heparin was studied by the toluidine blue test [21, 22] and silica by the spectroscopic method [23]. Scanning electron microscopy (SEM) was used to study the morphological characteristics of the silica-gel coating.

3. Results and discussion

The results from the cell culture tests and the cytotoxicity test [24] suggest that the acrylamide grafting does not alter the biocompatibility of the PLLA-co-CL. Both the contact and extract tests were carried out, and no significant differences between these results were observed [18].

3.1. Direct immobilization of heparin

Changing the reaction conditions, e.g. reaction time or temperature and pH of the incubation solution, could vary the amount of heparin immobilized on the grafted PLLA-co-CL. The attachment of heparin was best when acidic conditions (pH 4.5, acetic acid) were used (Table I). Up to 98 $\mu g\,cm^{-2}$ of heparin was immobilized on the surface of the PLLA-co-CL graft-polymer. The results were rather good even when only deionized water was utilized as a solvent. If the incubation solution is basic, the chemical structure of buffering solution must be taken into account. TRIS-buffer, which is a combined solution of TRIZMA HCl: tris[hydroxymethyl]aminomethane hydrochloride $(HOCH_2)_3CNH_{2x}HCl$ (Sigma Ultra/99.9%) and TRIZMA base: tris[hydroxymethyl] aminomethane $(HOCH_2)_3CNH_2$ (Sigma Ultra/99.9%), should be avoided because TRIS may react with heparin preventing the attachment to the polymer. By using phosphate buffer the attachment of heparin is possible, so absence of heparin in the TRIS case is not a matter of pH alone. Overall, the attachment of heparin to the surface was better in acidic conditions than in basic.

According to the thrombin test, the immobilized

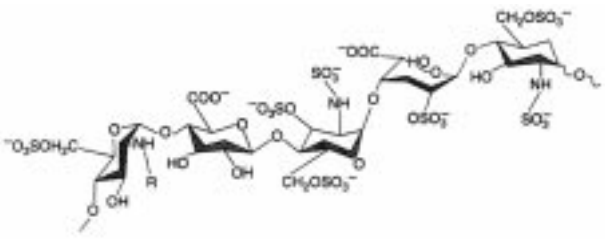

Figure 1 Chemical structure of the pentasaccharide segment (active site) of heparin molecule [14].

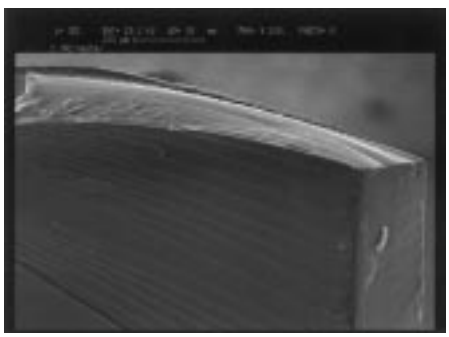

Figure 2 SEM image of the sol–gel-processed silica-gel coated polymer sheet.

TABLE I Effect of pH on the attachment of heparin on the surface of PLLA-co-CL polymer sheets

pH (reagents used for adjusting pH of the solution)	Heparin observed by toluidine test* (*mg*)	Amount of heparin at 1-cm^2 sheet (μg)
4.5 (acetic acid buffer)	23 ± 5	94
5.7 (deionized water)	9 ± 2	36
8 (TRIS-buffer)	–	–
7.8 (phosphate buffer)	3 ± 1	10

*The result is a mean value of three parallel measurements. Size of the sheets used for the measurements was 0.25 cm^2.

heparin retains its biological activity against thrombin formation. At least 76% of heparin, observed on the surface by toluidine blue test, showed biological activity as well. In the dissolution test, after 1 week in simulated body fluid at 37 °C, heparin was still immobilized because no heparin was released as estimated by the toluidine blue test or by the thrombin test.

3.2. Silica gel immobilized heparin coating

SEM pictures of the grafted surfaces showed that a uniform silica-gel coating about 0.3 μm thick was obtained with the dipping technique. Cracking of the silica-gel layer was minimal after bending sheets several times to 90 °C (Fig. 2). Heparin released from the silica coatings during the dissolution test retained its anticoagulant activity. The releasing rate of heparin follows that observed for silica [19]; after 1 week half of the immobilized heparin was released.

4. Conclusions

Electron beam radiation, as a method to produce radicals, and grafting, to attach a reactive polymeric layer on the surface of polymer, provide a useful combination when different reactive polymer surfaces are needed. Heparin, as an example of a biologically active molecule, was successfully attached on the top of a polymeric material produced by the grafting method. Traditionally, good attachment between the polymer surface and the biological molecule is achieved by coupling agents, for example glutaraldehyde or hexamethylene diisocyanate (HMDI), have been used [22, 25]. If radiation-induced grafting is used, no additional coupling agents are needed. The silica-gel coating obtained by dipping technique is uniform and heparin retains its biological activity.

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