Biotechnology Letters **25:** 381–385, 2003.

Conjugation of heparin into carboxylated pullulan derivatives as an extracellular matrix for endothelial cell culture

Kun. Na[1], Dongchul Shin[2], Kyusik Yun[3], Keun-Hong Park[1] & Kang Choon Lee[4,*]
[1]*College of Pharmacy, Chosun University, 375 Seoseok-Dong Dong-Ku, Kwangju 501-759, Korea*
[2]*Department of Materials Science Engineering, Kwangju Institute of Science and Technology, 1 Oryong-Dong, Buk-Ku, Kwangju 500-712, Korea*
[3]*BioElectronics Group, Devices & Materials Lab. LG Electronics Institute of Technology (LG Elite), 16 Woomyeon-Dong, Seocho-Ku, Seoul 137-724, Korea*
[4]*College of Pharmacy, SungKyunKwan University, 300 Chonchon-Dong, Jangan-Ku Suwon City 440-746, Korea*
**Author for correspondence (Fax: +82-62-228-3742; E-mail: kclee@skku.ac.kr)*

Received 22 August 2002; Revisions requested 17 September 2002; Revisions received 2 January 2003; Accepted 3 January 2003

Key words: endothelial cell, heparin, immobilization, pullulan, smooth muscle cell

Abstract

A carboxylated pullulan, for use as a structural material for a number of tissue engineering applications, was synthesized and conjugated with heparin. By immobilization of heparin to pullulan, endothelial cells (ECs) attached on the heparin-conjugated pullulan were more aggregated than when attached to other pullulan derivatives. Attachments were 50, 45, 49, and 90% for a polystyrene dish, pullulan acetate, carboxylated pullulan, and heparin-conjugated pullulan, respectively. Heparin-conjugated pullulan inhibited the proliferation of smooth muscle cells (SMCs) *in vitro*. Heparin-conjugated pullulan material can thus be used for the proliferation of vascular ECs and to inhibit the proliferation of SMCs.

Introduction

Advances in our knowledge of biochemical interactions at the cell surface have led to structure elucidation of ligand molecules that bind to cell surface receptors and influence cell behaviour. In many cases, the development of novel materials for *in vitro* and *in vivo* biotechnology applications will include these ligands (Barrera *et al.* 1993). The fabrication of these complex materials would be improved by the development of surface engineering techniques that permit the facile immobilization of high densities of ligands without degradation of either ligand or material. To date, the vast majority of ligand coupling to material is through covalent attachment of ligands to the bulk polymer.

In the present report, we have studied the incorporation of heparin into carboxylated pullulan acetate and assessed the suitability of the resulting materials as substrates for endothelial cell growth. Heparin is a linear polysaccharide containing repeating units of six sugar residues, each consisting of an alternating sequence of sulfate derivatives of *N*-acetyl-D-glucosamine and D-iduronate. Heparin was chosen primarily due to its powerful anticoagulant action (Machovich *et al.* 1975). It is also a component of the extracellular matrix of blood vessels and promotes endothelial cell growth *in vitro* (Garner *et al.* 1999). These properties make it an extremely useful molecule to incorporate into materials that could ultimately be used in the fabrication of vascular prostheses that require a non-thrombogenetic surface is required. Development of biomaterials that pomote the growth of endothelial cells is of special interest in the fields of vascular prosthetics and stent technology. The incorporation of heparin into carboxylated pullulan and the ability of such materials to support endothelial cell growth were thus the major focus of the present study. In this study, the natural polymer of carboxylated pullulan was used as a backbone material because

of its processability and biocompatibility. Heparin-conjugated carboxylated pullulan should be a useful biomaterial because of its suitability as a substrate for endothelial cell growth.

Materials and methods

Materials

Heparin, sodium salt (140 U mg^{-1}), of an average molecular weight of about 12 000 Da, was purchased from Pharmacia Hepar Co. (Franklin, OH). Pullulan (Mw; 200 000) was purchased from Hayashibara. Medium 199, Modified Eagle Medium (MEM), fetal bovine serum (FBS), bovine serum albumin (BSA), basic fibroblast growth factor (bFGF), collagenase, collagen type I, streptomycin, and penicilin sulphate were purchased from Gibco. Trypsin-EDTA, succinic anhydride, triethyl amine, formamide, pyridine, acetic anhydride, 4-dimethylaminopyridine (DMAP), dicyclohexyl carbodiimide (DCC), and hydroxylsuccinimide (HOSU) were purchased from Sigma. Dimethyl sulfoxide (DMSO)-d_6 was purchased from Aldrich Chemical Co. and DMSO and methanol were purchased from Showa Chemical Co. (Tokyo, Japan).

Pullulan acetylation

Pullulan, 2 g, was stirred vigorously in 20 ml formamide at 50 °C until dissolved. Six ml pyridine and 15 ml acetic anhydride were added and the mixture was stirred at 54 °C for 48 h. Pullulan acetate (PA) was obtained after precipitation from 200 ml water. The synthesized PA was identified using FT-IR and NMR. NMR study gave following information for the degree of acetylation, 1.27 unit of acetyl group per repeating unit of pullulan in one glucose.
IR (KBr): 3402 (s, OH), 2954 (m-s, CH_2), 1752 (vs, C = O) and 1375 (s, CH_3)
^{1}H NMR (DMSO-d_6): $\delta = 1.94 - 2.09$ (d, $-COCH_3$) and 3.96–5.23 (m, saccharide ring).

Synthesis of pullulan acetate (PA) grafted with heparin

Pullulan acetate, 5 g, was dissolved in a mixture of 200 ml dried 1,4-dioxane and 2.2 g succinic anhydride in a flask. The reaction mixture was stirred for 24 h at room temperature. The product was dissolved in 300 ml carbon tetrachloride to remove unreacted succinic anhydride. The degree of carboxylation was 0.54 unit of carboxyl group per one glucose unit of PA.

Heparin (30 mg), DCC (250 mg), and HOSU (150 mg) were added to 40 ml dried DMSO containing 1 g carboxylated PA, and allowed to react for 24 h at room temperature. After 24 h, reactant mixtures were filtered, and then dialyzed using a dialysis tube (molecular weight cutoff 12 000) against distilled water for 3 d. The samples were then lyophilized using a freeze-drier. To remove the unreacted material, lyophilized samples were dissolved in DMSO and then dialyzed with distilled water. The full process was repeated 3 times. The heparin immobilized PA was characterized by IR and ^{1}H-NMR spectrophotometer. The degrees of substitution (DS), defined as the number of heparin groups per 100 anhydroglucose units of heparin-PA were determined by ^{1}H-NMR and GPC, and the resulting DS of heparin-PA derivative was 16.7. The chemical structure of the heparin immobilized PA is shown in Figure 1.
IR (KBr): 3327 (s, NH), 2852 (m, OCH_3), 1751 (vs, C = O) and 1375 (s, CH_3)
^{1}H NMR (DMSO-d_6): $\delta = 1.95 - 2.07$ (d, $-COCH_3$), 2.73–2.89 (m, CH_2 CH_2), 3.49–3.67 (d, OCH_3), and 3.96–5.2 (m, saccharide ring).

Cell culture of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated by a collagenase dispersion method and maintained in Medium 199 supplemented with 20% (v/v) fetal bovine serum, 60 μg endothelial growth supplement ml^{-1}, 10 μg heparin ml^{-1}, 100 U penicillin ml^{-1}, and 100 μg streptomycin ml^{-1}. HUVEC were sub-cultivated after treatment with trypsin-EDTA and grown on collagen-coated tissue culture plastic. Cells were then seeded directly in the same culture medium to an approximately 1.25×10^4 cm^2 to heparin-conjugated pullulan derivative cover glass (prepared by a dip coating method) and then cultured in Medium 199 in 5% CO_2 and 95% air at 37 °C. The grown cell number and cell viability were evaluated by Trypan Blue and heamacytometer.

Smooth muscle cells from Sprague–Dawley rat aorta explants were isolated. Cells were grown in 75 cm^2 culture flasks at 37 °C with 5% v/v CO_2 in Modified Eagle Medium (MEM) supplemented with 2% (w/v) L-glutamine and 10% fetal bovine serum.

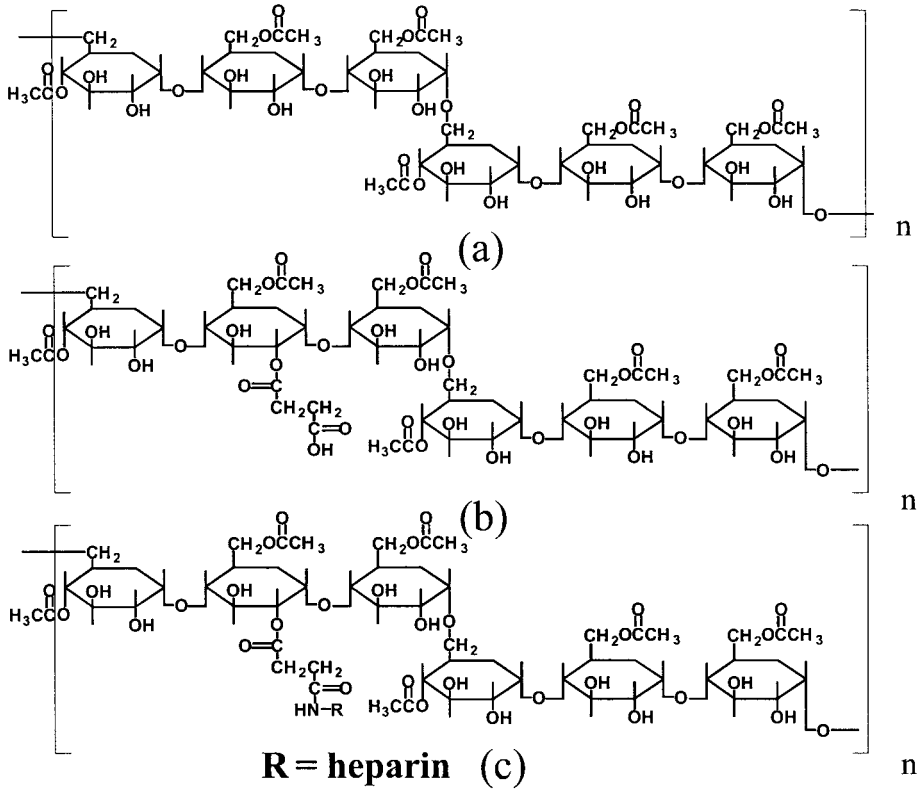

Fig. 1. Chemical structures used in this study. (a) Pullulan acetate (PA), (b) carboxylated pullulan acetate (PA-COOH), and (c) heparin-conjugated pullulan acetate.

Results and discussion

Cell growth of endothelial cells (ECs) attached to polymer-coated glasses

A suspension of ECs in medium was seeded onto several types of glass, taking care to distribute the cells evenly across the substrate. Attachment characteristics of endothelial cells to the pullulan acetate (PA) membrane, carboxylated PA membrane, and heparin-conjugated PA membrane were studied, and the results are shown in Table 1. A comparison of PA membrane and carboxylated PA membranes revealed that the cell attachment on heparin-conjugated membranes was better than that on PA and carboxylated PA membranes. Attachment to the polystyrene dishes was 50%. For PA, carboxylated PA, and heparin-conjugated membrane, the attachments were 45, 49, and 90%, respectively. This suggests that cell attachment is promoted by the presence of heparin ligands.

We also cultured ECs on heparin-conjugated membrane as well as other types of membranes. The growth characteristics of endothelial cells on various surfaces over a period of 72 h were recorded (data not shown). Confirming this generally observed behaviour, the ECs on the heparin-conjugated membranes exhibited more active growth than on the PA and carboxylated PA membranes. Effectively increased growth of ECs on the PA-heparin coated surfaces was observed for 24 and 72 h, while there was no significant increased growth on control and other pullulan derivatives. Presumably, initial attachment of ECs is relatively non-specific, whereas subsequent cell growth depends on more specific molecular interactions. It means that

Table 1. Cell growth of endothelial cells (ECs) attached to various surfaces.

Sample	Cell attachment (%)
Control (polystyrene dish)	50 ± 5
Pullulan acetate (PA)	45 ± 3
Carboxylated PA	49 ± 5
Heparin-conjugated PA	90 ± 2

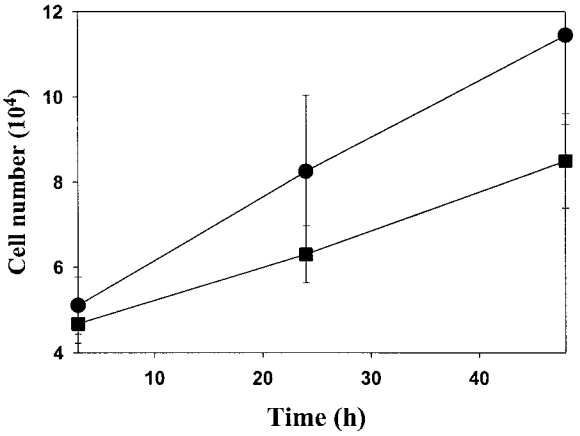

Fig. 2. Synergistical effect of bFGF on endothelial cell proliferative potential on heparin-conjugated membranes. (●): In the presence of bFGF, and (■): in the absence of bFGF. The data represents the experimental data (mean ± SD) ($n = 5$).

ECs attached to PA-heparin surfaces mediated by heparin receptors subsequently grew faster than those attached to other membranes.

Synergetical effect of bFGF on EC proliferation on heparin-conjugated surfaces

The *in vitro* effect of basic fibroblast growth factor (bFGF) in heparin-conjugated surfaces on the proliferative potential of ECs was evaluated by measuring the population of ECs during culture for up to 48 h (Figure 2). bFGF is a potent mitogen that stimulates proliferation, migration, and differentiation of cells of mesenchymal and neuroectodermal origins (Folkman & Klagsburn 1987). The EC population on the heparin-conjugated surfaces with bFGF was much larger than that of heparin-conjugated surfaces without bFGF, indicating that the incidence of ECs on heparin-conjugated surfaces was markedly enhanced in the presence of bFGF. bFGF has a strong affinity for glycosamingoglycan heparin, a property that has greatly facilitated its purification and characterization (Klagsbrun & Baird 1991, Yayon *et al.* 1991). For *in vitro* cell culture, it was reported that the addition of bFGF and heparin to a cultured medium synergetically enhances proliferation of ECs in organisms such as humans, dogs, and rats (Rifkin *et al.* 1989). Therefore, the addition of bFGF and heparin-conjugated surfaces may provide advantages such as enhanced cellular proliferative and migrative activities, due to the presence of bFGF.

Table 2. Effects of soluble heparin on inhibition of interaction between heparin-conjugated membranes and ECs.

Sample	Cell attachment (%)	
	Without heparin	With heparin
Control (polystyrene dish)	50 ± 5	46 ± 5
Pullulan acetate (PA)	45 ± 3	42 ± 3
Carboxylated PA	49 ± 2	45 ± 5
Heparin-conjugated PA	88 ± 4	58 ± 9

Table 3. Cell growth of smooth muscle cell (SMC) attached to various surfaces.

Sample	Cell attachment (%)
Control (polystyrene dish)	53 ± 5
Pullulan acetate (PA)	52 ± 3
Carboxylated PA	58 ± 5
Heparin-conjugated PA	12 ± 2

Effects of soluble heparin on inhibition of interaction between heparin-conjugated membranes and ECs

A suspension of EC cells in growth medium was seeded with soluble heparin onto various polymer-coated membranes, taking care to distribute the cells evenly across the membranes. The soluble heparin in the culture medium and heparin-conjugated membranes competed with each other for interaction with cells (Table 2). Despite the absence of any cells of other types, EC cells supplied with heparin evidenced little attachment to heparin-conjugate membranes. These results indicate that the attachment of EC cells was suppressed by the treatment with soluble heparin on heparin-conjugated membranes. Treatment of EC cells with heparin strongly blocked the attachment onto a heparin-conjugated matrix. This means that the heparin interacted in advance with heparin binding receptors on the cell surface. Therefore, heparin-conjugated substrata are considered to lose their specific binding sites for EC cells because of their occupation by treatment of heparin. This indicates that the heparin of PA-heparin copolymers specifically interact with heparin receptors on EC membrane.

Attachment of vascular smooth muscle cells (SMCs) on PA-heparin

In contrast to heparin-conjugated membranes, SMCs exhibit no significant reduction of cell attachment on PA and carboxylated PA surfaces, whereas ECs exhibit a 90% reduction of cell attachment on heparin-conjugated membranes (Table 3). Moreover, on heparin-conjugated pullulan surfaces, vascular SMCs exhibited a rounded morphology, and though all cells were well attached, the SMCs showed a stellate appearance. SMCs, in contrast to ECs, were able to spread enough to establish a cellular network on the PA or carboxylated PA membranes. The growth inhibition seen on the heparin-conjugated membranes may have resulted in part from the reduction in spreading, since cell spreading has been shown to correlate with DNA synthesis in some cell types (Hansen *et al.* 1994). This result indicates that polysaccharide surfaces can influence the attachment and morphological characteristics of vascular cells *in vitro*.

Acknowledgement

This work was supported by Korea Research Foundation (2001-003-F00236).

References

Barrera DA, Zylstra E, Lansbury PT, Langer R (1993) Synthesis and RGD peptide modification of a new biodegradable copolymer system: poly(lactic acid-co-lysine). *J. Am. Chem. Soc.* **115**: 11010–11011.

Folkman J, Klagsburn M (1987) Angiogenic factors. *Science* **235**: 442–447.

Garner B, Georgevich A, Hodgson AJ, Liu L, Wallace GG (1999) Polypyrrole-heparin composites as stimulus-responsive substrates for endothelial cell growth. *J. Biomed. Mater. Res.* **44**: 121–129.

Hansen LK, Moonney DJ, Vacanti JP, Ingber DE (1994) Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle to promote hepatocyte growth. *Mol. Biol. Cell* **5**: 967–975.

Klagsbrun M, Baird A (1991) A dual receptor system is required for basic fibroblast growth factor activity. *Cell* **67**: 229–231.

Machovich R (1975) Mechanism of action of heparin through thrombin on blood coagulation. *Biochim. Biophys. Acta* **412**: 13–17.

Rifkin DB, Moscatelli D (1989) Recent development in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* **109**: 1–6.

Yayon A, Klagsbrun M, Esko J, Leder P, Orintz DM (1991) Cell surface, heparin-like molecules are required for binding of basic growth factor to its high affinity receptor. *Cell* **64**: 841–848.