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The effect of electrostatic heparin/collagen layer-by-layer coating degradation on the biocompatibility

Jialong Chen[a,b,c,d,*], Nan Huang[b,**], Quanli Li[a], Chun H. Chu[c], Jun Li[d], Manfred F. Maitz[e]

[a] Stomatologic Hospital & College; Anhui Medical University, Key Lab. of Oral Diseases Research of Anhui Province, Hefei 230032, China
[b] Key Laboratory of Advanced Technologies of Materials, Ministry of Education, Southwest Jiaotong University, Chengdu 610031, China
[c] Faculty of Dentistry, the University of Hong Kong, 34 Hospital Road, Hong Kong, China
[d] College of Pharmacy, Anhui Medical University, Hefei 230032, China
[e] Leibniz Institute of Polymer Research Dresden, Max Bergmann Center of Biomaterials Dresden, Dresden 01069, Germany

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ABSTRACT

Electrostatic layer-by-layer coatings of heparin and collagen have been suggested before to improve the biocompatibility of blood-contacting devices. However, to our knowledge, there have been no systematic studies about the effect of degradation of this coating on its biocompatibility, anticoagulant properties and the cyto-compatibility. The purpose of this study was to design an in vitro experiment in this regard that can assess the degradation behavior and the biocompatibility change of the coating. The coating degradation in physiological saline (PS) under static and dynamic condition was monitored by DR-FTIR, SEM, AFM and water contact angle, moreover, heparin densities on the topmost surface and the release heparin every day were measured by toluidine blue O (TBO) assay. The results showed that the degradation rate of the coating in is much faster under flow and shear conditions than during static incubation, and only very limited collagen and heparin remain on the surface after 15 days incubation in dynamic condition. With the degradation, the hemocompatibility of the coating got worse, especially when incubated under dynamic conditions. The degradation products of the coating do not lead to coagulation but behave -as heparin- anticoagulant. The compatibility of the coating to endothelial cells improved within 15d incubation in static medium, but it for degradation under dynamic conditions, it improved for 5d but at 15d incubation, it was almost as low as for the bare substrate. These results highlight the necessity for appropriate testing of newly developed coatings not only in the initial state but also after extended exposure to a physiological ambient.

1. Introduction

Many cardiovascular and other blood contacting biomedical devices have been applied widely in recent years, including for example stents, artificial heart valves and vascular grafts [1]. However, biomaterial-induced thrombosis remains one of the most significant complications of vascular implant devices, leading to the loss of function by thrombotic occlusion or causing thromboembolism. Such complications may be reduced by further improvement of the hemocompatibility of the material surface [2].

Over the past decades, numerous efforts have been dedicated to enhancing the hemocompatibility by surface modification. Heparin is clinically the most commonly used anticoagulant drug and has also been widely used as an anticoagulant coating on the blood-contacting material surfaces by covalent immobilization or electrostatic adsorption [3–6]. Covalent immobilization makes a stable linking of the molecule on the surface for long time, but reduces its bioactivity [7,8]. Conversely, electrostatic adsorption retains the bioactivity, but weak molecular interactions lead to facile exchange and "stripping" of the adsorbed molecule in a short time [9].

The layer-by-layer assembly technique, LBL, enables the formation of complex multilayer films relatively simply through the sequential adsorption of oppositely charged polyelectrolytes. It has

* Corresponding author at: Anhui Medical University, Stomatologic Hospital & College, Key Lab. of Oral Diseases, Research of Anhui Province, Stomatologic Hospital & College, Hefei, Anhui 230032, China.
Tel.: +86 551 65161183; fax: +86 551 65161183.
** Corresponding author. Tel.: +86 28 87600625; fax: +86 28 87600625.
E-mail addresses: jialong_dt@126.com (J. Chen), huangnan1956@163.com (N. Huang).

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the unique advantages of high loading capacity, enhanced mechanical robustness, convenience in tailoring micro- and nanostructures, and the ability to integrate various functionalities into one film [9]. In past two decades, this technique became very popular in the field of biomedical research and there has been growing interest in the use of multilayers consisting of natural polyelectrolytes which are biocompatible, biodegradable and less toxic. On account of their biocompatibility and nontoxicity, the LBL coatings constitute a rapidly expanding field with potential applications: preparation of biomimetic films [10], drug release vehicles [11], bioactive coatings either by drug incorporation or by the use of the intrinsic properties of the polyelectrolytes [12,13] or build-up of cell adhesive or nonadhesive films [14,15]. Despite the incorporation of precise functionalities into the LBL coatings, the transition from stability to disintegration for these coatings in physiological ambiance is rapid, and it does not appear to be feasible to control the degradation rate of such decomposition [16,17]. To our knowledge, the precise functionalities of these coatings rely on their physicochemical properties, and these change with coating degradation. In addition, the time of persistence of these functionalities determine the therapeutic efficiency. But until now, the effect of degradation on the precise functionalities of the LBL coatings has not been studied systematically.

If the LBL coatings are to be used in a biomedical application, the physiological environment present in vivo must be taken into consideration. This will largely depend on the nature of the fluid in contact with the coated material in its specific location [5]. Some researchers evaluated the degradation behavior of the LBL coating used for cardiovascular system in static medium but not in dynamic [11,13]. In general, the static system has higher throughput and greatest ease of use but yields less-detailed information, while the flow-based test is more difficult to set up but is closest to physiology if one is interested in the dynamics of release in the vasculature [18]. In dynamic condition, shear force between the medium and the surface of the coating play an important role in the coating degradation. Herein, the difference of the LBL coating degradation behaviors between static and in dynamic condition needs to be studied.

Due to the highest negative charge density, heparin has been widely used in LBL technique for preparing anticoagulant coatings which could retain its bioactivity and release heparin for an extended time [12–15]. Because heparin can inhibit migration and proliferation of endothelial cells (EC), which could achieve sustained haemostasis and inhibit smooth muscle cell (SMC) migration and proliferation [19,20], some extracellular matrix proteins have been integrated into heparin-modified films by LBL technique for enhancing EC-compatibility [12,14,15,21]. Collagen, as one of extracellular matrix proteins, strongly promotes proliferation, differentiation, migration of cell [22], meanwhile, it contains a large amount of the alkaline amino acid lysine that gives the protein a positive net charge at pH 4 [23]. We recently prepared collagen/heparin (Col/Hep) coatings with thromboresistant effect and rapid re-endothelialization on the titanium surface by LBL technique [12,15]. It is well known that the LBL-multilayer gradually degrades due to the weak electrostatic interaction; as re-endothelialization is a long-term process, it is necessary to observe, how the biocompatibility is affected by the degradation of the coating. Moreover, the effect of degradation products on the coagulation system needs to be evaluated to exclude undesired biological response to these degradation products. These issues are typically neglected for LBL coatings.

Therefore in continuation of our previous study we analyze herein the effect of degradation of the heparin/collagen coating on the biocompatibility and the effect of the degradation products on the coagulation system.

2. Materials and methods

2.1. Materials

Commercial pure titanium (Ti) was purchased from Baoji Nonferrous Metal Co. Ltd. (Shanxi province, China). Poly-L-Lysine (PLL, 15–30 kDa) and Collagen type I were purchased from Sigma (USA). Unfractioned heparin with >150 U/mg was purchased from Santa Cruz biotechnology, INC (Shanghai, China). Other chemicals were all A.R grade agents.

2.2. Preparation of collagen/heparin multilayers

Collagen (Col) was dissolved in 0.2 M acetic acid at a concentration of 2.5 mg/ml. Heparin (Hep) was used at a concentration of 5 mg/ml in deionized water. PLL was used at 2.5 mg/ml in deionized water.

Ti substrates ($1 \times 1 \times 0.2\,cm^3$) were polished to a reflective mirror-like surface. The specimens were ultrasonically cleaned first in a detergent solution, then in acetone, ethanol, and finally in deionized water. After soaked in 5 M NaOH solution at 60 °C for 24 h, the cleaned specimens were soaked in deionized water at 80 °C for 8 h, and then were denoted as **TiOH**.

The NaOH treated samples were immersed in PLL solution for 30 min, thus obtaining a stable positive charged precursor layer to initiate the LBL self-assembly process. The substrates were dried with a stream of nitrogen. Multilayer films were deposited on the titanium substrates treated with steps above using a dip-coating technique at room temperature. First, the substrates were dipped in the heparin solution for 15 min, rinsed with deionized water, dried with a stream of nitrogen and then dipped into the collagen solution for 15 min, followed by rinsing with deionized water and drying with a stream of nitrogen. The cycle was repeated 30 times to obtain the desired film thickness, with the outermost layer heparin, and was denoted as **TC**. After the final assembly cycle, the substrates were dried with a stream of nitrogen.

The specimen surfaces were characterized by scanning electron microscopy (SEM; QUANTA 200, FEI, Holland), atomic force microscopy (AFM, SPI3800N Seiko, Japan), diffuse reflectance fourier transform infrared spectroscopy (DR-FTIR, ST-IR20SX, NICOLET Co. Ltd., USA) and water contact angel and surface energy determination (DSA100, KRÜSS, Germany).

2.3. Biodegradation of the Col/Hep coating

After placed into centrifugal tube, the samples with Col/Hep coating were immersed in 5 ml of physiological saline (**PS**, 0.9% NaCl), and then were incubated under static or dynamic (orbital shaker at 120 rpm) conditions at 37 °C. All of immersion solutions were collected for quantification of the heparin release every day and fresh PS was added to simulate the real in vivo condition. After incubating 5 days and 15 days, the samples were rinsed with deionized water and, respectively denoted as **PS-5d and PS-15d**. Then samples were characterized by SEM, AFM, DR-FTIR and water contact angel and surface energy determination.

2.4. Heparin quantification

The heparin density on the topmost surface of all the samples and the released heparin from the Col/Hep coating into the immersion solutions were characterized by toluidine blue assay (TBO assay) [24]. First, a series of heparin standard solutions (0–20 μg/ml) was prepared in PS. The heparin standard solutions (0.1 ml) or the samples with Col/Hep coating or the collected immersion solutions were then reacted with 1 ml toluidine blue solution (0.0005 wt%) in a centrifuge tube. The samples were