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Electrolytic deposition of hydroxyapatite/calcium phosphate-heparin/gelatin-heparin tri-layer composites on NiTi alloy to enhance drug loading and prolong releasing for biomedical applications

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ABSTRACT

Although the initial success of bare metal stents has significantly reduced the restenosis rate for percutaneous transluminal coronary angioplasty from 35% to 25%, the biological mechanism such as vascular smooth muscle cells (VSMCs) proliferation and neo-intimal hyperplasia still may induce in-stent restenosis (ISR). Therefore, some drug eluting stents have been introduced to reduce ISR. In this study, heparin (Hep) combined with calcium phosphate (CaP) and gelatin (Gel), without any additive or solvent, is co-deposited on hydroxyapatite (HA) coated NiTi alloy in order to enhance the drug load and the sustaining release for promoting the hemo-compatibility of NiTi substrate. Polarization tests were carried out in several solutions to investigate deposition mechanisms. Heparin contained composite coatings were characterized by X-ray diffractometry, Field emission scanning electron microscope, Fourier transform infrared spectroscopy, toluidine blue colorimetric assay, UV–visible spectrometer and kinetic clotting tests. The consequences indicate that heparin accompanied respectively with CaP, and Gel through ionic bonds can be loaded on the NiTi alloy. The porous post-HA coating can dramatically enhance the heparin content from 148 for the single layer coating (CaP-Hep) to 325 μg/cm^2 for the tri-layer coating (HA/CaP-Hep/Gel-Hep), also resulting in the heparin release duration from 1 to > 35 days, supposed to meet the requirement to prevent the proliferation of VSMCs. Both the drug content and releasing time are remarkable. As the result of clotting tests *in vitro*, drug loaded composite coatings reveal good anticoagulant property which is proportional to the cumulative content of drug release in an hour, indicating no denaturalization of heparin found during the electrochemical process.

1. Introduction

Since percutaneous transluminal coronary angioplasty (PTCA) was first conducted by Andreas Grüntzig in 1977 [1], this revolutionary treatment has become the most common procedure to deal with the cardiovascular disease. However, the high frequency about 30–40% of restenosis after PTCA [2,3] is the most important problem which needs to be resolved.

The stent was introduced by Charles T. Dotter in 1964 [4] and the first stent implantation surgery on human patient was performed by Puel and Sigwart in 1986 [5]. As a scaffold to maintain the lumen of blood vessel, stents successfully prevent restenosis by eliminating acute recoil and vascular remodeling. Although the restenosis rate has been significantly reduced to 20%–30% because of the initial success of bare metal stents (BMS), the in-stent restenosis (ISR) does happen due to the biological mechanism such as smooth muscle cell proliferation and neo-intimal hyperplasia.

Due to the insufficiency of the BMS, researchers shifted attention to other possible ways to improve the efficiency of stents, such as looking for more appropriate stent materials to meet the requirement of mechanical properties needed or obtaining the much more biocompatible and hemo-compatible surfaces by the surface modified technologies [4]. Among all of the methods, the invention of drug eluting stents (DES) takes a major step in the history of stents. Based on the basic mechanism and different pathways leading to ISR, the anti-proliferative, immunosuppressive and antithrombotic drugs should be considered.

The first two successfully commercialized DES are sirolimus-eluting Cypher™ stent and paclitaxel-eluting TAXUS™ stent [5], which localized the release of these anti-proliferative and immunosuppressive agents to inhibit the migration and growth of smooth muscle cell. Even so the DES can impressively reduce the ISR [6] and replace the BMS because

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of its short to mid-term security. Still there is doubt about its long-term efficacy.

In the porcine model which was published by the father of Cypher stent, unfortunately, the smooth muscle cell proliferation is more rigorous than the BMS group after 3 to 6 month [7]. First, it seems that the endothelialization and healing of the vessel wall are delayed by eluted drugs. Secondly, the residual polymer may induce a hypersensitivity reaction which is also related to the late-stent restenosis (LSR) [8–11]. Therefore, using biodegradable polymers as drug carriers or polymer-free surfaces without any additive or solvent would be a favorable option.

The most well-known biodegradable polymers used for medical application are poly-lactic acid, poly-glycolic acid and poly-lactic-*co*-glycolic acid. These polymers can mix with drug compounds to control release and totally degrade, avoiding the polymer-remained problem. There are numerous devices of these kinds under investigations in recent years [12]. However, the additives or solvents used in the synthesize process result in certain adverse effects. Besides, some researchers concluded non-polymer thin film coated stent with heparin (Hep) is non-inferior to commercial zotarolimus-eluting stent with polymer, with lower fibrin and inflammation score in porcine coronary restenosis model [13].

Heparin, with the powerful anticoagulant activity and highly negative charge, is a good candidate for clinical usage. One of the most important applications of heparin is modifying the stent surfaces to improve hemo-compatibility of materials and prevent thrombus formation [14]. Besides, John J. Castellot Jr. had revealed the structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells (VSMCs) based on that both anticoagulant and non-anticoagulant heparin species can inhibit the proliferation of VSMCs *in vivo* and *in vitro* [15].

Besides, in order to inhibit effect on VSMCs, the methods such as physical adherence and chemical immobilization are typical. Physical adherence is coating heparin by its negative charge to bind with the positive-charged surface through ionic attraction. Chemical immobilization is using the crosslinking reagent to covalently immobilize heparin on other polymers [16]. Both advantages and disadvantages are discovered in the two methods. On one hand, the physical-adhered heparin, due to the weaker bonding than the covalent bond in the chemical process, make the drug be depleted much more quickly. On the other hand, the chemical methods reduce or inhibited the activity of heparin [17,18].

Regarding to the problems mentioned above, the anti-thrombosis medical devices without synthesized polymers, additive or solvent would be required to avoid the adverse effects. Gelatin (Gel), with both carboxyl groups and amino groups, is a hydrolysis derivative of collagen. The amino groups could bind with protons when the pH value of solution is below the isoelectronic point of gelatin, making it a potential candidate to interact with heparin as well [19]. Hydroxyapatite (HA) is an excellent material as bone cement, coating for dental implants and being a carrier in drug release system because of its good biocompatibility and osteo-conductivity [20–22]. HA can also be used for artificial blood vessels and percutaneous devices, due to its non-toxicity, non-inflammatory, and non-hemolysis [23]. Recently, researchers focused on growth factors-loaded stents modified with hyaluronic acid and heparin for induction of rapid and tight re-endothelialization [24]. However, the amount of heparin immobilization is below 20.0 μg/cm^2.

The aim of this study is to fabricate the heparin sustaining release system to improve the hemo-compatibility of nitinol which has been applied to the cardiovascular stents or devices with the antithrombotic ability, without any additive or solvent. The multilayer coating containing porous HA, calcium phosphate (CaP), and gelatin combined with heparin is carried out by the electrochemical deposition to enhance the drug loading and prolong the releasing time. The coatings are characterized by the X-ray diffractometry (XRD), Field emission scanning electron microscope (FESEM), UV–visible spectrometer, Fourier transform infrared spectroscopy (FTIR), and *in vitro* clotting assays.

2. Materials and methods

2.1. Materials

Ni-Ti alloy disks with 1 × 1 cm and 0.5 mm thickness were used as substrates. Before electrochemical deposition, exposed metal surfaces were mechanically ground on 1200-grift SiC paper and then cleaned ultrasonically in ethanol and de-ionized water (D.I. water). Heparin sodium powders were purchased from Sigma-Aldrich H3149, Grade I-A, ≧180 units/mg, gelatin (Gel) from Fluka Chemie, Biochemica 48723 (bloom 160), Buchs, Germany.

2.2. Cathodic polarization tests and deposition

A mixed solution containing 0.042 M $Ca(NO_3)_2$ $4H_2O$ (SHOWA, Japan) and 0.025 M $NH_4H_2PO_4$ (SHOWA, Japan) was assigned to CaP solution. Another one containing 1.0% gelatin (SIGMA-ALDRICH, USA) was assigned to Gel solution. The solutions described above were also mixed with 800 IU/ml heparin (Hep) sodium salt (SIGMA-ALDRICH, USA) respectively to form CaP-Hep and Gel-Hep solutions for the heparin contained composite coating.

In order to investigate the deposition mechanism, the uncoated specimens were dynamically polarized in the CaP, CaP-Hep, Hep, Gel, and Gel-Hep solutions respectively, the former 3 by cathodic methods and the latter 2 by anodic ones, using a platinum foil as the counter electrode, a saturated Ag/AgCl the reference electrode, and the Ni-Ti disk the working electrode by an EG & G 273A Potentiostat and Power Suite software 352 (Princeton, USA). The pH values (pH meter; CYBERSCAN-500, USA), O_2 concentrations (O_2 meter; CLEAN, USA) and conductivity (conductivity meter; COSCAN CON6, USA) of electrolytes were measured and listed in Table 1.

After electrochemical analyses, the electrolytic deposition of HA on Ni-Ti alloy disks was carried out in CaP solution at 65 °C and applied potential −2.0 V (*vs.* Ag/AgCl) to derive the HA coated specimens [25], subsequently in the CaP-Hep solution at −2.0 V (*vs.* Ag/AgCl) at room temperature to derive Ha/CaP-Hep coated ones, and finally in the Gel-Hep solution at +1.5 V (*vs.* Ag/AgCl) to derived HA/CaP-Hep/Gel-Hep coated ones at room temperature as well to ensure that the drug is not affected [26].

2.3. Coatings characterization

Field emission scanning electron microscopy (FE-SEM, JSM-6700F JEOL, Japan) with working voltage 3 kV and working distances from 2.8 to 8.2 mm was used to observe the surface morphology of coatings before and after the drug releasing of several determined durations.

The related crystal structures were investigated by XRD (BRUKERS MXP-III, Germany) using 2.0 kW Cu Kα radiation source (λ = 1.5418 Å), voltage 40 kV, current 30 mA, glancing angle 2°, scanning rate 0.5°/min, and scanning range from 10 to 70°.

FTIR (BOMEM FTIR DA3.002*, USA) was used to examine the characteristics of chemical bonding. Samples were prepared by the KBr

Table 1
pH values, O_2 483 concentrations, and Conductivities of CaP, CaP-Hep, Hep, Gel, Gel-Hep solutions, and DI water.

	pH	O_2 concentration (ppm)	Conductivity (μS/cm)
CaP	4.05	7.02	10.2
CaP-Hep	4.28	9.05	10.72
Hep	6.16	10.7	899
Gel	5.52	1.15	208
Gel-Hep	6.14	1.21	1137
Distilled (DI) water	6.81	9.54	9.96