J Mater Sci: Mater Med (2011) 22:507–514
DOI 10.1007/s10856-011-4235-5

Surface grafting of blood compatible zwitterionic poly(ethylene glycol) on diamond-like carbon-coated stent

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Received: 18 October 2010 / Accepted: 11 January 2011 / Published online: 30 January 2011

Abstract Blood compatibility is the most important aspect for blood-contacting medical devices including cardiovascular stents. In this study, the surface of nickel–titanium (TiNi) stent was coated with diamond-like carbon (DLC) and then subsequently grafted by using zwitterion (N^+ and SO_3^-)-linked poly(ethylene glycol) (PEG). We hypothesize that this coupling of zwitterion and PEG may significantly improve blood compatibility of DLC-coated TiNi stent. The surface modified TiNi stents, including PEG-grafted stent (DLC-PEG) and zwitterionic PEG-grafted one (DLC-PEG-N-S) were the main focus on the tests of surface characteristics and blood compatibility. The zwitterionic PEG derivatives were obtained from a series of chemical reactions at room temperature. The results exhibited that as compared to the DLC-PEG, the hydrophilicity was much better with DLC-PEG-N-S and significantly increased atomic percentage of oxygen and nitrogen proved the entity of zwitterions on the surface of DLC-PEG-N-S. Meanwhile, the adsorption of blood proteins such as, human serum albumin (HSA) and fibrinogen was found considerably down-regulated in DLC-PEG-N-S, due mainly to the protein-repellant effect of PEG and zwitterion. Microscopic observation also revealed that as compared with the other substrates without zwitterion, the degree of platelet adhesion was the lowest with DLC-PEG-N-S. In addition, DLC-PEG-N-S retained an extended blood coagulation time as measured by activated partial thromboplastin time (APTT). The present results suggested that surface grafting of zwitterionic PEG derivatives could substantially enhance the blood compatibility of TiNi-DLC stent. In conclusion, anti-fouling properties of PEG and zwitterions are expected to be very useful in advancing overall stent performance.

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1 Introduction

With the introduction of bare metal stent (BMS) and following drug-eluting stent (DES), it is obvious that they have revolutionized the way of medical treatment in the field of interventional cardiology and angiology [1–6]. However there are still many concerns: the late restenosis with BMS and very late thrombosis with DES [7, 8]. The late in-stent restenosis and stent-associated thrombosis that were induced by neointimal hyperplasia of smooth muscle cells (SMC) and blood coagulation are often fatal to the patients [9, 10]. Inserting of stent into an occluded vessel damages endothelial cell (EC) layers, which loses a normal function inhibiting the formation of blood clots, where the function is to regulate the infiltration of plasma components and inflammatory cells from the blood to the surrounding tissue to tackle injuries and infections [11, 12]. Unwanted accumulation of inflammatory cells in the space separating the endothelial cell layer (intima) from the SMC layer (media) result from EC layers' damage, and the increased inflammatory cell sends biochemical signals to the SMC which start to proliferate invading the lumen of vessel [13]. The resultant thrombus and restenosis in stent were generated by consequences of the clotting system activation and the excessive intima growth, respectively. To solve these problems, physical and chemical modifications of stent surface have been a routine for researchers.

For biomedical applications, diamond-like carbon (DLC) coating has been performed on various metal substrates such as titanium, 316 stainless steel, titanium oxide,

titanium nitride, cobalt–chromium alloy, and Nitinol alloys [14]. Researchers reported that DLC showed promising results as a candidate material to improve biocompatibility [15, 16]. Lee, et al. described that DLC coating reduced thrombogenicity by minimizing the platelet adhesion and activation [14]. However, several controversies remain in the effect of the DLC coating for biomedical applications. Mikhalovska et al. reported that carbon coating on metal substrates didn't demonstrate significant decrease in fibrinogen adsorption and platelet adhesion in comparison to the metal materials [17]. Airoldi, et al. showed that no difference in 6-month restenosis rate was observed when bare stainless steel (SS) and carbon-coated SS stents were inserted into coronary artery [18]. Also, Santin, et al. suggested diamond-like carbon (DLC) coating may not be able to prevent restenosis [19]. Bioinert materials are required to DLC-coated metal surface to dispel this controversy and complement a poor haemocompatibility or biocompatibility.

Bioinert materials, containing poly(ethylene glycol) (PEG) or zwitterion used to improve the blood compatibility, have demonstrated their capability in repelling the surface adsorption of proteins and other biomolecules [20–23]. PEG has accepted much attention as a candidate material which can enhance biocompatibility due to low interfacial free energy with water and inertness for protein adsorption and cell adhesion, high chain mobility, and steric stabilization effect [24]. The phosphorylcholine group with zwitterionic materials, along with hydrophilic PEG, also showed its antithrombogenicity and excellent resistance for protein adsorption [25]. Lee, et al. reported that the surface coverage of the nonspecific binding of a model protein absorbed on the gold surfaces grafted with alkanethiol containing three or five EG moieties is relatively 30 or 50 times lower than that of the surface without the EG moieties by surface plasmon resonance (SPR) spectroscopy, respectively [23]. The PEG-containing surface, which is grafted via surface-initiated atom transfer radical polymerization (SI-ATRP) of oligoethylene glycol methacrylate (OEGMA) from a solid substrate presenting atom transfer radical polymerization (ATRP) initiators, showed relatively lower surface coverage of the nonspecific binding of model proteins such as fibrinogen and lysozyme, as compared with that of the surface grafted with alkanethiol compounds containing the linear EG moieties by SPR measurements [26, 27]. The long chain zwitterionic poly(sulfobetaine methacrylate) (PSBMA) and hydrophilic/neutral POEGMA surfaces grafted via ATRP showed more excellent anti-biofouling effects than that of the surface with self-assembled monolayers (SAMs) of alkanethiols in reducing bacterial adhesion and suppressing biofilm formation [28]. In addition, PEG-containing compounds have found their utility to prolong the circulation of bloodstream due to retarding the clotting time in the blood contacting areas [24, 25, 29]. A combination of PEG and zwitterionic compounds may be very useful to enhance the overall blood compatibility of the blood contacting devices because the surface of stent is definitely required to have excellent blood compatibility. In the previous study, Han et al. have showed that the surface grafting of bioinert PEG or zwitterionic PEG derivatives could improve the blood compatibility of two-dimensional (2D) TiNi substrates [30–32]. To draw a synergistic effect of both PEG and zwitterion, this work designed a direct grafting of zwitterionic PEG on the surface of TiNi stent, a three-dimensional (3D) substrate. Therefore, the primary interest is to evaluate whether this undocumented combination can be effective in improving blood compatibility.

In this study, the zwitterionic PEG-grafted stent was fabricated through the covalent binding between –NCO group of PEG derivative and the hydroxyl group on the stent substrate. The PEG derivatives, such as OCN-PEG-NCO, OCN-PEG-N, and OCN-PEG-N-S (zwitterionic PEG) were synthesized in a series of chemical reactions and then subjected to characterized using ^{1}H-NMR, ATR-FTIR, and elemental analyzer (EA). For further improvement of blood compatibility, the bare metal TiNi stent was pretreated with diamond-like carbon (DLC) by plasma-assisted chemical vapor deposition (PACVD). Once the stent-DLC served as a control and surface treatment was followed by oxygen plasma onto stent-DLC, PEG grafting, and finally zwitterionic PEG grafting, respectively. We hypothesize that the treatment of zwitterionic PEG on the DLC-coated stent would have an additive benefit in enhancing blood compatibility over stent-DLC control. To demonstrate the hypothesis, the adsorption of human serum albumin (HSA) and fibrinogen, platelets adhesion, and blood coagulation time are evaluated, respectively. Four types of stent substrates were used: stent-DLC control, stent-DLC-OH, stent-DLC-PEG, and stent-DLC-PEG-N-S.

2 Materials and methods

2.1 Materials

TiNi alloy (Nitinol) stents (diameter 6 mm, length 6 cm) were obtained from Taewoong Medical Co. (Kimpo, Korea). Poly(ethylene glycol) (PEG, MW: 2,000), hexamethylene diisocyanate (HDI), *N,N*-dimethylethanolamine (DMEA), human serum albumin (HSA), and human fibrinogen were purchased from Aldrich (USA). Dibutyltin dilaurate (DBTDL) and propane sultone (PST) were obtained from TCI (Japan). Platelet concentrates (PC) and fresh frozen plasma (FFP) was obtained from The National Red Cross (Korea). Lactate dehydrogenase (LDH) was

purchased from Takara (Japan). Other chemicals were of analytical grade and used as received.

2.2 Synthesis of zwitterionic PEG

The synthesis of zwitterionic PEG was performed according to the literature reported previously [31]. To make an isocyanate PEG, PEG (6.25×10^{-3} mol) was reacted for 45 min with HDI (13.1×10^{-3} mol) dissolved in 10 ml of toluene under nitrogen at 40°C in the presence of tin catalyst (0.2% DBTDL). When one side of the isocyanated PEG (OCN-PEG-NCO) was then reacted with DMEA, a tertiary amine group, $–N–(CH_3)_2$, was introduced to generate a tertiary-aminated NCO-PEG (NCO-PEG-N). Finally, zwitterion ($–SO_3^-$ and $-N^+$) was coupled by using a ring-opening reaction of PST with NCO-PEG-N, which resulted in a zwitterionic PEG (OCN-PEG-N-S). The four types of PEG derivatives, HO-PEG-OH, OCN-PEG-NCO, OCN-PEG-N, and OCN-PEG-N-S were analyzed using ^{1}H-NMR (JNM-LA 200WB FT-NMR, 200 MHz, JEOL, Japan). The four materials were dissolved in $CDCl_3$ and chemical shifts were adjusted with reference to an internal standard tetramethylsilane (TMS).

2.3 DLC coating of TiNi stent

DLC coating on the TiNi stent was carried out using 13.56 MHz RF plasma-assisted chemical vapor deposition (PACVD). The stents were placed on the water-cooled cathode, where the RF power was delivered through the impedance matching network. Prior to the deposition of DLC, the samples were sputter-cleaned with Ar plasma for 15 min. Purged with the precursor gases of benzene and diluted silane (SiH_4:H_2 = 10:90), DLC was deposited onto the TiNi stent under a bias voltage of −400 V and deposition pressure of 1.33 kPa. The thickness of DLC layer was 0.55 ± 0.01 μm as determined by an alpha step profilometer.

2.4 Zwitterionic PEG grafting on TiNi-DLC stent

Before the surface grafting of the PEG (OCN-PEG-N) and the zwitterionic PEG (OCN-PEG-N-S) on the TiNi-DLC stent, the stent was placed in the plasma chamber and subject to oxygen plasma discharge for the oxidation of the surface. The plasma generator carried a frequency of 13.56 MHz (RF-GEN, IDT Eng. Co., Korea). The specifications of the plasma treatment were as follows: plasma power of 260 W, exposure time of 10 min, and oxygen pressure of 0.2 m Torr. The plasma-treated stent was vacuum-dried for 24 h, resulting in an oxidized TiNi-DLC stent. As the oxidized TiNi-DLC stent was immersed in 2 ml toluene added with 0.2 g zwitterionic PEG (OCN-PEG-N-S) and 0.002 ml magnesium stearate, these mixtures were stirred for 24 h at 100 rpm at 40°C in the pyrostat tank (BS-20, JEIO TECH, Korea), and followed by vacuum-drying for 24 h. As a result, a zwitterionic PEG-grafted TiNi-DLC (Stent-DLC-PEG-N-S) was obtained. Meanwhile, in a separate procedure, the hydrophilic OCN-PEG-N was grafted on the oxidized TiNi-DLC stent to make a PEG-grafted TiNi-DLC (Stent-DLC-PEG), following the same procedure as used for zwitterionic PEG grafting.

2.5 Surface analysis

To evaluate the effect of surface treatments, the chemical structures of stent-DLC control, stent-DLC-OH, stent-DLC-PEG, and stent-DLC-PEG-N-S were analyzed using attenuated total reflection-fourier transform infrared (ATR-FTIR, IFS 66 spectrometer, Bruker, Germany). Post-treatment changes on the chemical elements were also investigated using electron spectroscopy for chemical analysis (ESCA, S-Probe Surface Science, Mountain View, CA, USA). The relative atomic percentage was calculated from the peak areas. Water contact angle, an indicator of surface wettability, was determined using an optical bench-type contact angle goniometry (Digidrop, GBX Scientific Instrument, France). Once a drop was placed on the flat NiTi-DLC substrate, direct microscopic measurement of static contact angle was then carried out in triplicate and their average values were calculated. Surface morphology of the modified samples was observed using scanning electron microscopy (SEM; S-2500C, Hitachi, Japan).

2.6 Protein adsorption and platelet adhesion

After control and surface-treated stents were hydrated for 30 min in phosphate-buffered saline (PBS), they were then immersed in human fibrinogen (0.3 mg/ml) and in HSA (3 mg/ml), respectively, for 1 h at 37°C with a gentle shaking in water bath. The samples were rinsed three times in PBS and then treated with 5% sodium dodecyl sulfate (SDS) for 24 h to detach the total proteins physically adsorbed on the surface. The amount of isolated protein was measured using bicinchoninic acid (BCA) protein assay. In addition, for platelet adhesion test, the total number of platelets in the PC was adjusted to 1×10^5 by adding FFP. Once the stents were incubated for 1 h in the platelet suspension (2 ml) at 37°C, they were washed three times with PBS, and fixed in 2% glutaraldehyde for 1 h. The stents were dehydrated sequentially with the use of aqueous 50, 70, 80, 90, and 100% ethanol solution. The morphology of the platelet-adhered surfaces was visualized using SEM. The tests on

protein adsorption and platelet adhesion were repeatedly performed in triplicate, respectively.

2.7 In vitro blood coagulation time

Surface-modified TiNi stents were inserted in a tube after washing with PBS solution, and then added to FFP of 2 ml, being reacted at 37°C for 1 h. The stents were removed in the tube after the reaction, and then the time arrived in a half of best turbidity of the FFP solution according to blood coagulation was evaluated using activated partial thromboplastin time (APTT) analyzer (Sysmex, model CA-50, Japan). Namely, both FFP solution of 50 μl after removing the substrates and the actin solution of 50 μl were mixed in a reaction tube at 37°C for 1 min, followed by adding 50 μl of $CaCl_2$ solution. After reacting of the resultant solution at 37°C for 3 min, the coagulation time kit was measured in triplicate.

2.8 Statistical analysis

As the data were expressed as means ± standard deviation (SD), statistically significant difference between each group was determined using Student *t* test. When *P* value was lower than 0.05, the difference was considered statistically significant.

3 Results and discussion

The synthesized PEG derivatives were individually characterized by ^{1}H-NMR, ATR-FTIR, and XPS, respectively [31, 37]. The chemical structures of PEG-grafted compounds were identified using ^{1}H-NMR (Fig. 1). The methyl proton of HDI was confirmed as the two peaks appeared at 1.3–1.5 ppm after the first reaction. The proton of N–$(CH_3)_2$ in DMEA was found at 2.3 ppm. For the final reaction, the proton of N–$(CH_3)_2$ was shifted from 2.3 to 3.3 ppm and the methyl protons of PST were identified at 2.3, 3.0, and 4.4 ppm, respectively. Analyzed by using ATR-FTIR (Fig. 2), the characteristic peak of -NCO in OCN-PEG-NCO appeared at 2,270 cm^{-1}, as compared with HO-PEG-OH. Specific peaks of OCN-PEG-N and OCN-PEG-NCO were undetected due to the superposition of their absorption modes. In the case of OCN-PEG-N-S, the specific peak distinguished from that of OCN-PEG-N was observed at 1,037 cm^{-1}. Both theoretical and experimental values for carbon, hydrogen, nitrogen, and sulfur were found closely matched when the atomic percentages of PEG derivatives were determined by elemental analyzer

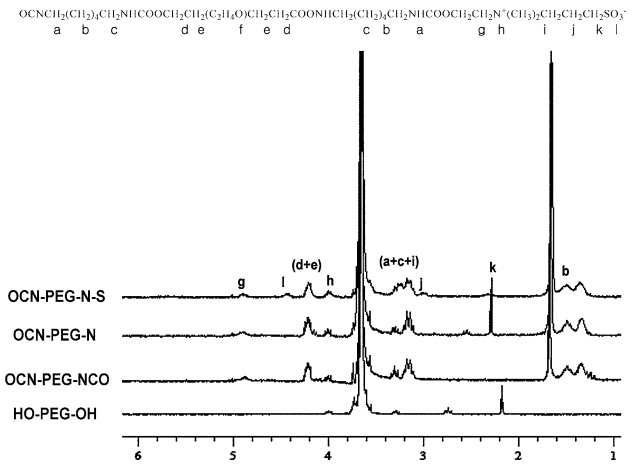

Fig. 1 ^{1}H-NMR spectra of HO-PEG-OH, OCN-PEG-NCO, OCN-PEG-N, and OCN-PEG-N-S

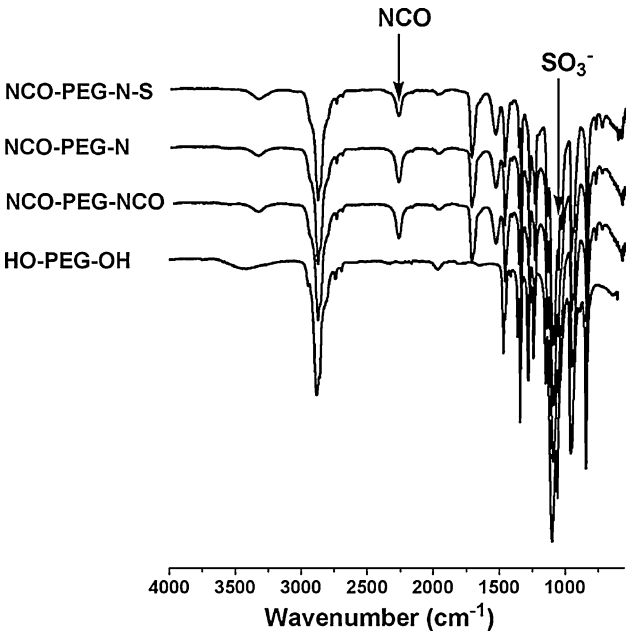

Fig. 2 ATR-FTIR spectra of HO-PEG-OH, OCN-PEG-NCO, OCN-PEG-N, and OCN-PEG-N-S

Table 1 Atomic percentage of the synthesized compounds of PEG derivatives

Material	At.% (X/Y)[a]			
	C	O	N	S
HO-PEG-OH	54.5/52.9	36.4/37.9	–	–
OCN-PEG-NCO	54.7/52.9	35.0/37.4	1.3/0.8	–
OCN-PEG-N	54.8/53.1	34.3/35.2	1.9/2.7	–
OCN-PEG-N-S	53.4/51.5	34.6/36.4	1.8/2.6	1.3/0.8

[a] *X* theoretical value, *Y* experimental one

(Table 1), indicating that the synthesis of PEG derivatives was completely carried out.

The procedural steps of surface modification of TiNi-DLC stent were progressed as presented below. First, hydroxyl groups were introduced by the treatment of oxygen plasma on the stent-DLC control, resulting in stent-DLC-OH. PEG moieties were then coupled with the stent-DLC-OH substrate that was reacted with the isocyanate -NCO group of OCN-PEG-N. In a separate work, the zwitterionic PEG (OCN-PEG-N-S) was also grafted onto the stent-DLC-OH substrate in a same way, producing stent-DLC-PEG-N-S. The modified surface characteristics were evaluated using ATR-FTIR (Fig. 3). The stent-DLC-OH carried a distinct peak of the C–C bonds over 3,000 cm^{-1}, which originated from O_2 plasma treatment on the stent-DLC control [33, 34]. In addition, the C–O peak in stent-DLC-OH was also found near 1,100 cm^{-1} due to an oxygen effect from O_2 plasma activation. In the case of stent-DLC-PEG, the peaks of $–CH_2–$ and $–CH_3–$ were strongly observed at less than 3,000 cm^{-1}, which were resulted from methyl and methylene groups of the PEG [27]. The unique IR peak of SO_3^- in stent-DLC-PEG-N-S was identified at 1,028 cm^{-1}, indicating that the zwitterionic PEG was grafted onto stent-DLC-OH substrate. On the other hand, the impact of plasma treatment was obvious as the contact angles were determined (Table 2). The contact angles of stent-DLC-OH decreased from 90° (control) to 78° due to the grafted hydroxyl group after plasma treatment. A significant decrease of the contact angle of stent-DLC-PEG-N-S seemed to be due to the added hydrophilicity induced from both PEG moieties and sulfobetaine of N^+ and SO_3^-. The surface properties of stent were also characterized by analyzing the atomic percentage of elements by ESCA (Table 2). While the

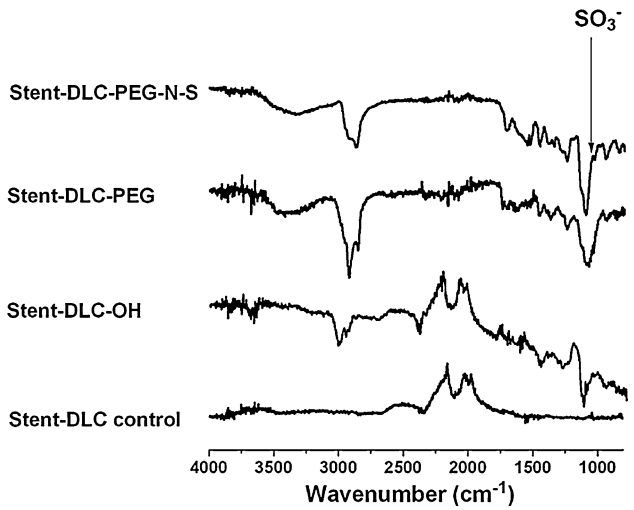

Fig. 3 ATR-FTIR spectra of stent-DLC control, stent-DLC-OH, stent-DLC-PEG, and stent-DLC-PEG-N-S

Table 2 ESCA atomic percentage and static contact angle for four types of stent-DLC substrate

Substrate	At.%				Contact angle (degree)
	C	O	N	S	
Stent-DLC control	88.58	11.42	–	–	90 ± 4
Stent-DLC-OH	87.12	12.89	–	–	78 ± 3
Stent-DLC-PEG	78.10	21.69	0.21	–	40 ± 3
Stent-DLC-PEG-N-S	75.33	22.02	2.46	0.18	25 ± 2

nitrogen in stent-DLC-PEG came from a urethane linkage generated by the covalent binds, the detection of sulfur in stent-DLC-PEG-N-S supported the entity of successfully grafted zwitterionic PEG.

Meanwhile, the amounts of adsorbed human blood proteins on the surface-modified stents were measured (Fig. 4). The results displayed that the total amount of HSA

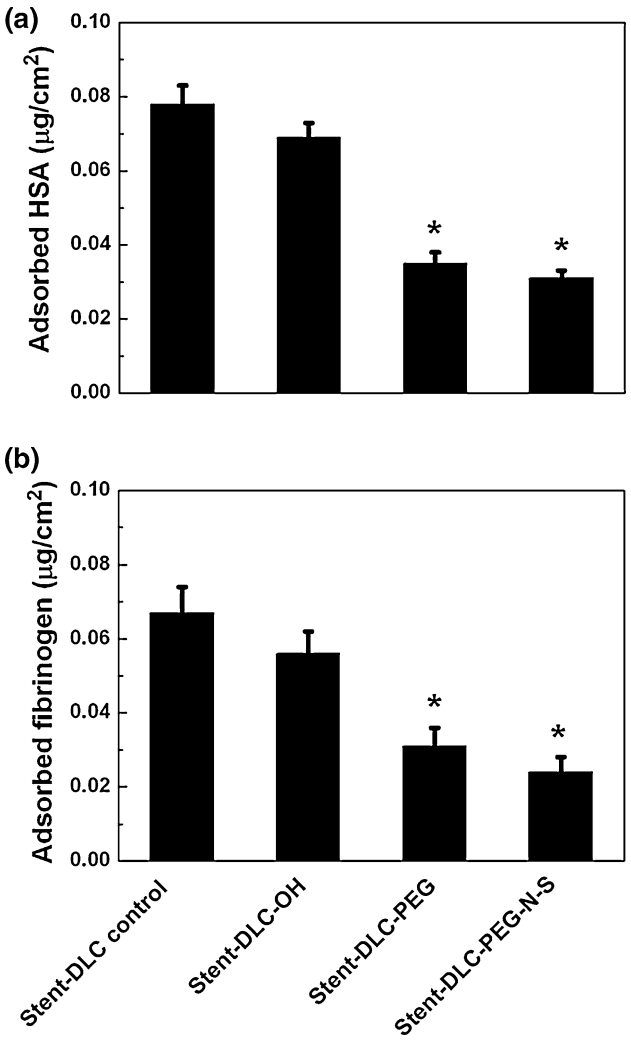

Fig. 4 Amount of **a** HSA and **b** fibrinogen adsorbed on stent-DLC control, stent-DLC-OH, stent-DLC-PEG, and stent-DLC-PEG-N-S ($P < 0.05$)

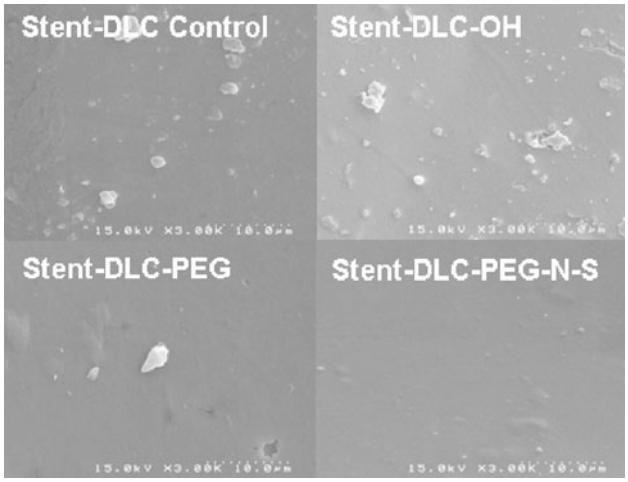

Fig. 5 Platelets adhesion by SEM (3,000×) for stent-DLC control, stent-DLC-OH, stent-DLC-PEG, and stent-DLC-PEG-N-S

and fibrinogen decreases considerably as the PEG biomolecule was grafted on the stent. The positive outcome of PEG- and zwitterionic PEG-grafted stents may come from the intrinsic anti-biofouling effect of PEG and zwitterions, as compared with stent-DLC and stent-DLC-OH [28, 35]. Those data demonstrate that the compounds containing PEG and zwitterion may significantly contribute to improving blood compatibility of stent. Platelet adhesion was also examined using the same test groups (Fig. 5). When the appearance of platelets adsorbed on the substrates was visualized by SEM, the amount of them seemed to be the least on the DLC-PEG-N-S. This is due mostly to a low affinity of platelets to the surface of zwitterionic PEG and thus this property of platelet can substantially enhance blood compatibility. In addition, the blood coagulation

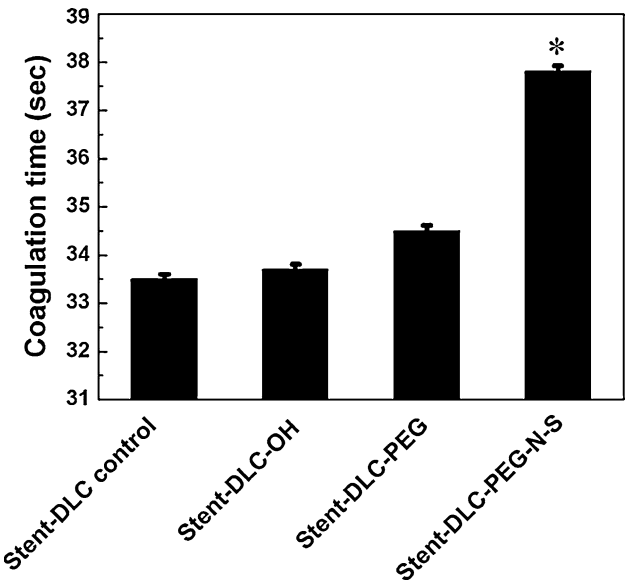

Fig. 6 Blood coagulation time of stent-DLC control, stent-DLC-OH, stent-DLC-PEG, and stent-DLC-PEG-N-S ($P < 0.05$)

time was determined by activated partial thromboplastin time (APTT) assay, which is the method to screen the deficiency of intrinsic coagulation factors such as fibrinogen and prothrombin [34, 36, 37]. Fig. 6 showed that zwitterionic PEG-grafted stent could deliver the longest time of coagulation. In the case of heparin as an anticoagulant, Jee, et al. reported that the coagulation time of polylactide (PLA)-heparin conjugate measured by APTT kit was prolonged when compared with PLA control (PLA; 31.8 s, PLA-heparin; 49.9 s) [38]. The reason that the clotting time of zwitterionic PEG-grafted stent prolonged as compared to the control could be inferred from the improved antithrombogenicity by zwitterionic interfacial molecular structure containing sulfonate groups [39]. The measurement of coagulation times of PEG derivative-treated surface stressed that the grafting of PEG and further zwitterions on the stents might enable them to carry more blood compatible properties.

4 Conclusion

PEG derivatives were chemically synthesized and their characteristics were identified by using ^{1}H-NMR, ATR-FTIR, and EA. Those derivatives were then successfully utilized for the surface modifications of DLC-coated TiNi stents. Since the primary interest of using zwitterionic PEG is to improve blood compatibility more effectively, the related experiments with human blood were carried out. The results showed that the level of protein adsorption and platelet adhesion significantly decreased with zwitterionic PEG-grafted stents, as compared to the control and other test groups. On the other hand, the coagulation time substantially increased with the use of zwitterionic PEG-grafted stents. These works once again repeated the intrinsic property of PEG and zwitterion as excellent blood compatible biomolecules. These anti-fouling biomaterials are expected to find a role in minimizing thrombosis induced by blood coagulation in blood-contacting medical devices, especially for bare metal stent (BMS) and drug-eluting stent (DES).

Acknowledgments This research was supported by a grant from the Fundamental R&D Program for Core Technology of Materials funded by the Ministry of Knowledge Economy, Republic of Korea (K00060-282) and by the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2010-0002170).

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