Rapamycin-loaded nanoporous $\alpha$-Fe$_2$O$_3$ as an endothelial favorable and thromboresistant coating for biodegradable drug-eluting Fe stent applications†

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Iron and its alloys can be potentially employed to fabricate advanced degradable cardiovascular stents due to their excellent mechanical and biocompatibility properties. However, their clinical applications are hindered by their inherent slow degradation rate, the formation of thrombosis and in-stent restenosis. In this study, vertically oriented and orderly arranged $\alpha$-Fe$_2$O$_3$ (hematite) nanotubes with diameters ranging from 30 nm to 70 nm were successfully fabricated on iron substrates using an anodic oxidation approach. These nanotubular coatings acted as drug depots by being loaded with anti-proliferation drug rapamycin to accelerate the re-endothelialization process and being coated by PLGA through a simple spin-coating process to control the drug release rate. The static immersion test showed that the 50 nm-Fe$_2$O$_3$ nanotube arrays displayed a faster corrosion rate than pristine Fe, and the PLGA coating effectively reduced the initial burst release of the loaded drug and extended the rapamycin release time to 30 days. The CCK-8 assay and immunofluorescence staining analysis results indicated that the endothelial cells (ECs) on the coated samples showed higher cell viability than the vascular smooth muscle cells (VSMCs), with possible outcomes to promote re-endothelialization and decrease VSMC proliferation. In addition, the surface modified iron exhibited very good hemocompatibility. The current findings suggested that fabricating rapamycin-loaded and PLGA coated Fe$_2$O$_3$ nanotubes on a pure iron surface may be a promising method to improve the corrosion rate and accelerate the re-endothelialization of the iron for biodegradable cardiovascular stent applications.

1. Introduction

In recent years, significant developments have been made in the field of biodegradable stents for treating cardiovascular diseases. Among them, biodegradable metallic stents have gained more interest than their polymeric counterparts because of their superior mechanical properties.

Fe-based and Mg-based alloys are two typical and widely investigated biodegradable metallic materials for fabricating biodegradable stents.1–3 Recently, Zn-based materials also emerged as alternative biodegradable stent materials.4–6 Compared with Mg-based and Zn-based alloys, Fe-based stents are more advantageous in terms of their comparable mechanical properties to 316L stainless steel (316L SS).2 In addition, iron is an essential element for the body, and it could reduce the proliferation rate of vascular smooth muscle cells.7 A period of 6–12 months is required for the vessels to undergo the remodelling process and then the stents are expected to degrade and to be eliminated from the patient after implantation for 12–24 months.2 Early in vivo experimental results demonstrated that the degradable iron stents could be safely implanted into the descending aorta of New Zealand white rabbits without significant inflammation, neointimal proliferation, or thrombotic events,8 and the pure iron stent strut still remained within the blood vessel even after 12 months.9 The slow degradation rate of Fe may result in the long term presence of this metallic stent. Compared with the vascular tissues, the metallic stents are much harder and more rigid, and their long term presence within the blood vessels may possibly cause damage to and irritations of the vascular tissues or induce undesired
biological reactions. Therefore, a faster degradation rate of iron stents is desirable for clinical applications to avoid the potential side effects that result from the slow degradation rate. Many methods have been exploited to produce iron-based materials with increased degradation rate, such as developing novel iron alloys\textsuperscript{10} or producing pure iron by using electroforming technique.\textsuperscript{11}

Surface engineering is an effective technique to mediate the biological and physicochemical properties of biomaterials. Iron can be patterned cost-effectively with vertically oriented \(\alpha\)-Fe\textsubscript{2}O\textsubscript{3} nanotubes through a simple and facile electrochemical technique for their potential applications as photocatalysts (similar to TiO\textsubscript{2}).\textsuperscript{12} Considering their application as biodegradable stents, pure iron with nanotubular topography has three advantages: (I) better cellular responses, (II) faster degradation rate, and (III) great potential for being exploited as drug-eluting stents. (I) A case in point is that, compared with the flat Ti surface, the adhesion and proliferation as well as motility of the endothelial cells are significantly improved on nanotubes with a diameter of 15 nm,\textsuperscript{13–15} indicating that nanotubular coatings can display strong stimulation of cellular activities. (II) Another example is that these nanotubes were expected to improve the cytocompatibility and enhance the anodic dissolution of the iron substrate by the Fe\textsubscript{2}O\textsubscript{3}/Fe interfacial micro-galvanic reactions.\textsuperscript{16} (III) The implantation of bare metal stent is always accompanied by two major complications, the delayed endothelialization of the stents and the proliferation of vascular smooth muscle cells (VSMCs), resulting in the formation of thrombosis and in-stent restenosis, respectively. To tackle this problem, the nanotubular structures could be exploited as drug-delivery depots\textsuperscript{17} to inhibit VSMC proliferation and prevent restenosis.\textsuperscript{18}

Fe\textsubscript{2}O\textsubscript{3} has been reported to be applied in the vascular stents.\textsuperscript{19,20} To the best of our knowledge, there are no studies attempting to evaluate the biocompatibility of Fe\textsubscript{2}O\textsubscript{3} nanotubes on pure iron and to explore the possibility of utilizing Fe\textsubscript{2}O\textsubscript{3} nanotubes as drug depots/carriers for their applications as degradable vascular stents. Therefore, in our study, Fe\textsubscript{2}O\textsubscript{3} nanotube arrays were prepared on iron and drug-loaded (rapamycin) by a simple dipping method. Rapamycin (also known as sirolimus), a carboxyl lactone–lactam macrolide, is a natural fermentation product produced by Streptomyces hygroscopicus\textsuperscript{21} and has been widely used as a cardiovascular drug due to its potent inhibitory effects on the proliferation and migration of smooth muscle cells, which was reported to remarkably reduce the restenosis rate to almost 0\%.\textsuperscript{20,22,23} Moreover, due to the open-structure, and the high drug loading amount and the concentration gradient of the coating, the release of the drug directly from nanotubes would be very rapid,\textsuperscript{24} which could not endow these drug-containing stents with desired properties (sustained drug-release time and effective drug-release dosage) for clinical practice. In order to alleviate those undesired effects, in our study, these nanotubes were further coated by PLGA films through a simple spin-coating process.

Inspired by TiO\textsubscript{2} nanoscale surface topography and high surface specific area for promoting re-endothelialization, in our study, we fabricated vertically oriented and orderly arranged, drug-loaded and polymer-coated \(\alpha\)-Fe\textsubscript{2}O\textsubscript{3} nanotubes for cardiovascular biodegradable drug-eluting stent applications. Their surface micro-structures, compositions, electrochemical properties, in vitro cyto-hemocompatibility, etc. were firstly well investigated, providing a promising candidate for the next generation of vascular materials.

2. Materials and methods

2.1 Materials

Fe foils (0.25 mm thickness, purity 99.9\%) were provided by Shandong Institute of Medical Instrument. Rapamycin, cholecystokinin-octapeptide (cck-8), 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), phallolidin (fluorescein isothiocyanate labeled) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 In situ fabrication of Fe\textsubscript{2}O\textsubscript{3} nanotubes

Fe foils (99.9\%) with a thickness of 0.25 mm were cut into 1 cm × 1 cm pieces and used as substrates for the fabrication of Fe\textsubscript{2}O\textsubscript{3} nanotube arrays. The samples were degreased by sonication in acetone and ethanol, followed by rinsing with ethanol and dried in a nitrogen stream. A schematic representation of the anodisation setup is shown in Fig. 1. In situ Fe\textsubscript{2}O\textsubscript{3} nanotubes were fabricated by electrochemical anodization in an ethylene glycol electrolyte containing ammonium fluoride and deionized water, the platinum foil served as a counter electrode, and pure iron foils were used as anodes. Iron nanotubes (FeNTs) with various diameters were obtained by adjusting potential (DC power supply, Agilent Technologies, 0–60 V) and duration at 20 °C. After electrochemical treatment, the samples were rinsed with ethyl alcohol, dried in a nitrogen stream, and subsequently annealed in air for 2 h at 500 °C with heating and cooling rates of 5 °C min\textsuperscript{-1} to obtain the crystallized and stable forms of the iron oxide nanotubes.

2.3 Drug loading

Rapamycin was dissolved in acetone at a concentration of 1 mg mL\textsuperscript{-1}. Rapamycin solution (200 \(\mu\)L) was dropped into PLGA films through a simple spin-coating process.

![Fig. 1 Schematic diagram for the in situ fabrication of Fe\textsubscript{2}O\textsubscript{3} nanotubes on pure iron by electrochemical anodization with Rapa loaded and PLGA coated structures.](image-url)
each Fe$_3$O$_4$ nanotube array substrate and dried at room temperature, which was denoted as Rapa. Polymer solutions of PLGA (2% (w/v) in acetone) and PLGA (2% (w/v)) with rapamycin (15 µg mL$^{-1}$) were prepared. 100 mL of solution was dropped on the surface and spin-coated at 5000 rpm for 1 min, and this procedure was repeated five times. The samples coated with PLGA and PLGA-rapamycin were denoted as PLGA and PLGA-Rapa, respectively.

### 2.4 Surface morphology, microstructure characterization and hydrophilic properties

The surface morphology of the samples was observed by field emission scanning electron microscopy (FESEM; S4800; Hitachi). The crystalline structures of the iron nanotube were identified using an X-ray diffractometer (XRD) (Rigaku DMAX 2400) with Cu Kα radiation. X-ray photoelectron spectroscopy (XPS; AXIS Ultra, Kratos Analytical Ltd) was employed to identify the surface composition of the film. Raman measurements were performed at 633 nm by using a Raman Imaging Microscope System (Renishaw 1000) in a backscattering geometry. The hydrophilic properties were investigated using the static water contact angles. Static water contact angles were measured at 25 °C, using the sessile drop method (Dataphysics Instrument, Germany). The mean value of four replicates was used as the final result for each sample.

### 2.5 Electrochemical corrosion behaviors of iron with Fe$_3$O$_4$ nanotube coatings

Electrochemical corrosion behaviors of the samples were investigated by electrochemical measurements. According to ASTM F 2129-08, a three electrode system was used to conduct electrochemical measurements in an electrochemical analyzer (CHI 660C, Shanghai CH Instrument Co., China), in which the reference electrode was saturated calomel and the counter electrode was platinum. Hank’s solution$^{15}$ with a pH value of 7.4 ± 0.1 was used. After the specimen was immersed in Hank’s solution for 1 h, the potentiodynamic polarization tests were carried out from −1100 mV to 0 mV (vs. SCE) at a scanning rate of 0.33 mV s$^{-1}$. Electrochemical impedance spectroscopy (EIS) was carried out by setting the DC voltage to the open circuit potential of the sample under test with an AC amplitude range of 10 mV to 10 mV and evaluated in the frequency range from 10$^{-2}$ Hz to 10$^{4}$ Hz. The corrosion rate was calculated according to ASTM G 102-89 by using the formula:

$$V_{corr} = \frac{K_1 I_{corr}}{\rho}, \quad CR = K_2 I_{corr,EW}$$

where $V_{corr}$ is the corrosion rate in terms of penetration rate, CR is the corrosion rate in terms of mass loss rate, EW is the equivalent weight, $K_1 = 3.27 \times 10^{-3}$ mm g µA$^{-1}$ cm$^{-1}$ year$^{-1}$ and $K_2 = 8.954 \times 10^{-3}$ g cm$^{-2}$ µA$^{-1}$ m$^{-2}$ day$^{-1}$. CR is given in mm year$^{-1}$ and $I_{corr}$ in µA cm$^{-2}$.

### 2.6 Drug release characterization

Colorimetry was used to quantify the rapamycin release amount from the samples by measuring the absorption values of the solution at 270 nm, with a calibration curve of the rapamycin concentrations varying from 0 to 25 µg mL$^{-1}$. Drug release from the drug-loaded and polymer-coated (PLGA and PLGA-Rapa) samples was investigated by immersing the samples in 10 mL of phosphate-buffered saline (PBS) for different times (1, 3, 5, 7, 10, 15, 20, 25 and 30 days), and the absorbance of the PBS extract was measured at 270 nm. The corresponding drug concentration was calculated based on the calibration curve obtained for the drug. Ultimately, the drug release percentage (wt%) was calculated from the amount of drug released into the PBS solution divided by the total amount of drug multiplied by 100.

### 2.7 Cytotoxicity evaluation

The cell viability of the endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) on the samples was evaluated by the cell counting kit-8 assay (CCK-8, Dojindo). According to the ISO 10993-5:1999, the extraction medium was prepared by using DMEM-serum-free medium with a surface area/extraction medium ratio of 1.25 cm$^2$ mL$^{-1}$ in a humified atmosphere with 5% CO$_2$ at 37 °C for 72 h, and the extraction medium was stored at 4 °C before the cytotoxicity test. Cells were incubated in 96-well cell culture plates at the density of 5 × 10$^4$ cells per mL of medium in each well for 12 h to allow the cell adhesion. DMEM was used as a negative control and DMEM containing 10% dimethyl sulfoxide was employed as a positive control. The medium was then replaced with 100 µL of extraction medium. After incubating the cells for 1 d, 3 d and 5 d, 10 µL of CCK-8 was added to each well and further incubated for 2 h at 37 °C. After that, the optical density (OD) of the supernatant was measured using a spectrophotometer (Elx800, Bio-Tek instruments) at a wavelength of 450 nm. The cell viability was calculated according to the following equation:

$$\text{Cell viability(%) } = \frac{\text{OD(test)} - \text{OD(blank)}}{\text{OD(negative)} - \text{OD(blank)}} \times 100\%$$

All experiments were carried out five times.

### 2.8 Immunostaining for actin

For the Fe$_3$O$_4$ nanotube coated samples, VSMCs and ECs were seeded on the pristine and nanotube modified Fe substrate at a density of 2.5 × 10$^4$ cells per cm$^2$. Cells were incubated in complete medium under standard culture conditions. After culturing for 6 h, cells were fixed in 4% paraformaldehyde in PBS. Subsequently, the cells were permeabilized with 0.5% Triton X-100 in PBS, blocked with 1% BSA in PBS for 30 min, and stained in Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA) for 60 min at room temperature. Samples were imaged using a Nikon C1si Spectral Confocal microscope. For the drug-loaded and PLGA coated samples, the extraction medium was prepared using DMEM-serum-free medium with a surface area/extraction medium ratio of 1.25 cm$^2$ mL$^{-1}$ in a humified atmosphere with 5% CO$_2$ at 37 °C for 72 h. Sterile glasses (Φ = 12 mm) were put into the 24-well plate, and ECs and VSMCs were seeded at a density of 2.5 × 10$^4$ cells per cm$^2$. Cells were incubated in complete media under standard culture conditions for 6 h and replaced by the extraction medium later.
After being cultured for 1 d and 5 d, cells were treated by the same procedures for staining and imaging.

2.9 Hemolysis test

Healthy human blood from a volunteer containing sodium citrate (3.8 wt%) at a ratio of 9 : 1 was taken and diluted with normal saline (4 : 5 ratio by volume). The samples were dipped in separate standard tubes containing 10 mL of normal saline that were previously incubated at 37 °C for 30 min. Then 0.2 mL of diluted blood was added to these standard tubes and the mixtures were incubated for 60 min at 37 °C. As a positive control for hemolysis, 0.2 mL of blood was diluted in distilled water, whereas saline diluted blood was added to an empty standard tube which served as a negative control. After this period, samples were removed and all the tubes were centrifuged at 800g for 5 min. The supernatant from each tube was transferred to a well in a 96-well plate where the absorbance was measured using a microplate reader (Bio-Rad 680) at 545 nm. The hemolysis rate was calculated as follows:

$$\text{Hemolysis rate} = \frac{\text{OD}(\text{test}) - \text{OD}(\text{negative control})}{\text{OD}(\text{positive control}) - \text{OD}(\text{negative control})} \times 100\%$$

where OD is the optical density at 545 nm.

2.10 Platelet adhesion

Platelet-rich plasma (PRP) was prepared by centrifuging whole human blood from a volunteer at 200g for 15 min. The PRP was dropped on the surface of the experimental samples and incubated at 37 °C. After 1 h, the samples were gently rinsed with phosphate buffered saline (PBS) to remove non-adherent platelets. They were then fixed in 2.5% glutaraldehyde solutions for 2 h at 4 °C, followed by dehydration in a gradient ethanol/distilled water mixture from 30% to 100% in 10% increments for 10 min each and finally freeze-dried for 2 d. After being sputter coated by gold, the surfaces of platelet attached samples were observed by SEM.

2.11 In vitro coagulation time measurements

Platelet-poor plasma (PPP) was prepared by centrifuging the whole human blood from a volunteer at 3000 rpm for 15 min. Then 0.5 mL of PPP was dropped onto the surface of the samples. After incubation for 15 min at 37 °C, the Activated Partial Thromboplastin Time (APTT) measurements were performed by adding 100 μL of incubated PPP solution to 200 μL of APPT reagent in a test tube, and then the time was measured using a coagulation instrument SYSMEX CA1500 (SYSMEX, JP). As for Thrombin Time (TT) measurements, 100 μL of incubated PPP was added to 100 μL of TT reagent in a test tube, and time was read. The average of five measurements was taken.

All the experimental protocols were approved by the Institutional Review Board (IRB) of Peking University and were performed in compliance with the relevant laws and institutional guidelines.

3. Results

3.1 Surface morphology and microstructures

Vertically highly ordered Fe$_2$O$_3$ nanotubes with average diameters of 30 nm (20 V, 30 min, 1% water content), 50 nm (30 V, 15 min, 2% water content), and 70 nm (50 V, 8 min, 3% water content) were successfully in situ fabricated on the Fe substrate at 20 °C and are shown in Fig. 2(a–c). In order to screen out the optimized parameters for fabricating Fe$_2$O$_3$ nanotubes, the effects of voltage, oxidation time, electrolyte temperature and water content on the formation of these nanotubes were systematically investigated and the results are presented in the ESL. All the resulting nanotubes were orderly and vertically oriented, and the diameters of nanotubes increased with the increase of applied potential. Post-heat treatment was conducted by annealing the prepared unstable amorphous samples at 500 °C in air for 2 h to obtain the crystallized and stable forms of the iron oxide nanotubes.

To explore the possibility of using these nanotubes as drug-carriers, Fe$_2$O$_3$ nanotubes (50 nm) were selected and loaded with rapamycin (200 μg cm$^{-2}$) by simply dropping the drug onto the nanotubes. The rapamycin aggregates were not observed in the side view of Fe$_2$O$_3$ nanotube arrays (Fig. 2d), indicating a uniform drug loading. Due to its good biocompatibility and biodegradability, PLGA was reported as an effective drug carrier and barrier layer with sustained drug release, such as bone morphogenetic protein-BMP$^{26,27}$ and antibiotics. So in order to mediate the drug release rate therein, these samples were further spin-coated by PLGA (Fig. 2e, with a thickness of about 20 nm) and PLGA-Rapa (Fig. 2f), respectively, with a similar SEM surface morphology. We also noticed that both the uncoated and PLGA coated nanotubular layers on the iron substrate showed good integrity after 5 min of ultrasonic treatment.

The crystalline structures of the as-anodized and annealed iron oxide nanotubes were examined by X-ray diffraction (XRD) (Fig. 3). Before the heat treatment, the anodized nanotube was amorphous$^{29}$ and crystallized into α-Fe$_2$O$_3$ (hematite) after being annealed at 500 °C in air for 2 h. The XRD peaks for the heated samples could be indexed to the (012), (104), (110), (113), (024), (116), (214) and (300) diffraction peaks of the

![Fig. 2](image_url) SEM images of Fe$_2$O$_3$ nanotubes with diameters of about 30 nm (a), 50 nm (b) and 70 nm (c), as well as the Rapa loaded (d), PLGA covered (e) and PLGA-Rapa covered (f) 50 nm Fe$_2$O$_3$ nanotube samples. The (a–c) insets show the cross-sectional images of the anodized samples and the (d–f) insets show the tilted side-view images of the drug loaded/PLGA coated samples.
$\alpha$-Fe$_2$O$_3$ at 23.8°, 32.8°, 35.2°, 40.6°, 49.2°, 53.9°, 62.3° and 64.8°, respectively. In addition, a very small amount of Fe$_3$O$_4$ (magnetite) was observed as the secondary phase, which was much less than that of $\alpha$-Fe$_2$O$_3$.

### 3.2 Surface chemistry and wettability

The chemical compositions of the Fe$_2$O$_3$ nanotubes, as well as the drug-loaded and PLGA coated ones, were investigated by XPS. In order to shed light on the effects of heat treatment on the nanotube composition, the samples with 50 nm diameter nanotubes before annealing were denoted as anodized in Fig. 4.

Three main peaks of Fe, C and O were seen in the XPS spectra of the pristine iron. The high content of the oxygen may be due to natural oxidation of iron in the atmosphere, and the adsorption of oxygen and carbon dioxide. After anodic oxidation (Fig. 4a), besides the feature peaks of Fe, O and C, a strong peak of F (10.12 at%) was also detected, which was derived from the migration of F$^-$ ions from the electrolyte containing ammonium fluoride to the anodized coatings. In addition, the carbon contents increased from 8.65 at% to 44.81 at%, which could be attributed to adventitious elemental carbon and the presence of ethylene glycol in the electrolyte. When the anodized samples were subjected to heat treatment, the C and F content decreased dramatically to 10.64–12.1 at% and 0.79–1.7 at%, respectively, on account of the decomposition of the solvent and fluoride at high temperature during the heat treatment. The F and C contents increased slightly, when the nanotube diameters became wider. The content of O increased significantly after annealing. From the high-resolution spectra of the Fe 2p (Fig. 4(a–1)), the binding energy of the doublet Fe 2p$_{3/2}$ and Fe 2p$_{1/2}$ shifted from 705.9 eV and 719.0 eV for the pristine iron to 711.4 eV and 724.7 eV for the as-anodized and annealed samples, indicating that pure iron was oxidized to iron oxide through anodization treatment. The variations in the Fe 2p XPS spectra of 30 nm, 50 nm and 70 nm may originate from the slight differences in the iron ionic bond characteristics in the coatings. This conclusion could also be verified by the corresponding O 1s spectra (Fig. 4(b–1)) with the binding energy at 530.4 eV from Fe–O. After being loaded with rapamycin, the content...
of the carbon increased obviously and iron decreased by 8%. This was due to the high content of carbon in the rapamycin. However, the nitrogen was not detected. According to the composition of rapamycin, the content of nitrogen in the rapamycin was about 1.5% which was too little to be detected. For the PLGA-Rapa covered sample, the carbon content increased due to the incorporation of rapamycin into the PLGA. From the high-resolution spectra of the O 1s (Fig. 4(b–I)), it is found that the Fe–O absorption peak was present in the Fe2O3 nanotubes and Rapa samples; and the peaks of C–O and C–O were detected for the PLGA and PLGA-Rapa coated samples. As for the C 1s high-resolution spectra of the PLGA and PLGA-Rapa covered samples, the absorption peaks were composed of three peaks which were assigned to –COOH, –CH2OH and –CH3.

Fig. 5 demonstrates the initial water contact angles (WCAs) of original as well as the surface-functionalized samples. The initial WCAs of original Fe plates were as high as 65.26 ± 2.12°, and decreased obviously to 20.74 ± 0.11°, 14.78 ± 0.29° and 11.3 ± 2.47° for 30 nm, 50 nm and 70 nm nanotubes, respectively. According to the classical Wenzel approach, the contact angle depends on the roughness factor and the surface chemistry. It was speculated that the Fe2O3 nanotubes formed by anodizing were partially hydroxylated with polar nature, leading to relatively small advancing contact angles. The initial WCA of the Fe2O3 (50 nm) nanotubes was 14.78 ± 0.29°, and increased to 18.31 ± 2.45° with loaded rapamycin. Rapamycin was a kind of macrolide which was insoluble in water and could reduce the surface hydrophilicity. By coating the samples with PLGA and PLGA-Rapa, their WCA increased to 69.33 ± 0.02° and 72.50 ± 0.18°, respectively. These results were consistent with previous reports. After being covered by PLGA (with a molecular weight of 15 kDa which mainly made up of ester bonds), the surface became smooth, leading to the increase of water contact angle. The PLGA coatings could endow the substrates, such as Mg-6Zn17 and titania nanotube arrays,17 with good cytocompatibility.

3.3 In vitro corrosion properties and cytocompatibility of the Fe2O3 nanotube coatings

In order to screen out appropriate Fe2O3 nanotube coatings for drug-loading, the effects of the Fe2O3 nanotube coatings on the corrosion behaviors of the iron substrate were investigated by both the electrochemical method in Hank's solution and the immersion test in the cell culture medium. Compared with those of the original Fe, the OCP values of the nanotube specimens shifted toward more negative values with initial minor fluctuations and reached the average potentials of −0.589 V, −0.605 V and −0.586 V after immersion for 60 min (Fig. 6(a1)). According to the Nyquist plots in Fig. 6(a2), the diameters of the semicircle for the nanotube samples were smaller in comparison with that of the Fe in Hank's solution, which suggested the increase of the sample corrosion rate, with a sort order of 70 nm > 30 nm > 50 nm > Fe. As for the potentiodynamic polarization curves shown in Fig. 6(a3), the corrosion current densities (Icorr) of all the nanotube samples were about one order higher than those of the pristine Fe, which could be attributed to the significant increase of the specific area of Fe2O3 nanotubes. In addition, as the main degradation products of pure iron, the presence of the corrosion product layer (porous Fe2O3 deposits) on iron might enhance the anodic dissolution of iron, due to the adsorption of intermediate species such as Fe(i) ions and Fe(n) ions, and the resultant self-catalytic corrosion effect.

The degradation behavior of nanotube modified iron was further evaluated by measuring the concentrations of iron ions released from the samples after immersion in cell extraction medium for 3 d, as shown in Fig. 6(b). Clearly, the concentrations of iron ions released from the Fe2O3 nanotube samples were higher than those of the pristine iron, especially for 50 nm samples, indicating that anodization of iron could accelerate its degradation rate. However, in terms of the corrosion rate, the result of the immersion test was not consistent with that of the electrochemical test. It was speculated that this discrepancy resulted from the poor mobility of the media in the nanotubes. To further study the stability of the nanotubes, the surface morphologies of the samples after immersion in DMEM were observed, as shown in Fig. 6(c). It can be seen that the nanotube walls were thickened and the microstructure of the nanotubes was well maintained, suggesting the relatively stable properties of the Fe2O3 nanotubes in the cell culture medium.

Fig. 6(d) shows the in vitro cell viability of ECs and VSMCs cultured in the extraction media of the pristine and Fe2O3 nanotube modified samples for 1, 3 and 5 d, respectively. The cell viability of both cells on the Fe2O3 nanotube samples was significantly higher than that of the pristine iron group, but no obvious difference could be seen for the Fe2O3 nanotube samples with different diameters. Comparing the response of ECs and VSMCs to that of the iron nanotubes, for all the samples after being incubated for 3 d and 5 d, the viability of ECs was slightly higher or comparable to that of VSMCs, except for the 50 nm group after being cultured for 5 d (with an EC viability of 82.4 ± 4.6% and a VSMC viability of 61.6 ± 4.5%), suggesting that the extraction media containing ferric ions were favorable for EC growth. Immunofluorescence staining was also carried out to investigate the cell adhesion and migration behavior. As indicated in Fig. 6(e), compared with the flat pure iron, more cells were observed on the nanotube coated samples after 6 h of culturing, especially for the 50 nm groups, indicating that the 50 nm nanotubes could induce favorable biological responses in terms of cell adhesion. However, much more pronounced filopodial extensions with longer configuration were observed...
on 50 nm nanotubes. In contrast, almost no protrusion of filopodia can be observed on 70 nm nanotubes. An additional aspect noteworthy to mention is that the shape of endothelium on 50 nm nanotubes was elongate but not on 70 nm nanotubes and flat, suggesting enhanced cell migration activity on 50 nm nanotubes. The adhesion behaviors of these cells were also evaluated by SEM observation, which could be seen in Fig. S5 in the ESL.† This result revealed that the nanotubes induced strong cell proliferation and migration, especially in the case of 50 nm nanotubes for endothelium but not for smooth muscle cells. Cheng et al.59 prepared biodegradable Fe–Fe₂O₃ composites and their extracts showed no cytotoxicity to ECV304 and L929 cells, but significantly suppressed the viabilities of VSMCs, which shows similar results to our study.

Combined with the results of electrochemical analysis, the immersion test and the in vitro cytocompatibility evaluation of the Fe₂O₃ nanotube coated Fe, the 50 nm Fe₂O₃ nanotubes were selected and loaded with rapamycin for potential drug eluting applications.

3.4 Rapamycin release profiles and in vitro cytocompatibility evaluation of the rapamycin-loaded samples

The accumulated drug release profiles of the samples with and without a polymer film (PLGA and PLGA-Rapa) are depicted in Fig. 7. The release efficiency at various time intervals was tested. On the first day, the burst release of the uncoated sample was about 37.15%, and it dropped to about 16.71% for the coated samples. This implied that the PLGA and PLGA-Rapa coating could effectively reduce the initial burst release of the loaded drug. For the uncoated sample, the release kinetics can be described in two phases, a high percentage (92%) of the drug release during the initial 7 d and followed by a plateau with slow release over the following days. The burst release could be explained by the high concentration gradient within the nanotubes. For the PLGA and PLGA-Rapa covered samples, the release rates were lower. On day 10, the loaded rapamycin was almost completely released from the uncoated nanotubes, and only about 68.36% drug was released from the coated samples, indicating that the PLGA coating could effectively reduce the initial burst release of rapamycin and endow the drug-loaded Fe₂O₃ nanotubes with a long and sustained drug release.

Fig. 6 Chemical analysis of pristine and Fe₂O₃ nanotube coated Fe samples in Hank’s solution (a): open-circuit potential vs. time curves (a1), and Nyquist (a2) and anodic polarization plots (a3). The ferric ion concentrations (b) and the corresponding SEM morphologies (c) of Fe₂O₃ nanotubes with 30 nm (c1), 50 nm (c2) and 70 nm (c3) diameters after being immersed in DMEM for 3 d. Cell viability of ECs (d1), and VSMCs (d2) cultured for 1, 3, and 5 d in the extraction media of the samples. Immunofluorescence images (e) of cytoskeletal F-actin stains for ECs and VSMCs after incubation for 12 h on the prepared samples.
release time, which may be required for effectively inhibiting vascular cell hyperplasia.\textsuperscript{17,38} Compared with PLGA, the incorporation of rapamycin into the PLGA (PLGA-Rapa) could further increase the drug release amount and time. The inhibitory effects of rapamycin on the proliferation of cells were concentration and time dependent,\textsuperscript{39} and we expected that the PLGA-Rapa would have a better inhibitory effect on the cell growth.

Fig. 8 shows the \textit{in vitro} cell viability of ECs and VSMCs cultured in the extraction media of the Fe\textsubscript{2}O\textsubscript{3} nanotubes (50 nm) and rapamycin loaded, PLGA covered and PLGA-Rapa covered samples for 1, 3 and 5 d, respectively. Both ECs and VSMCs cultured in the extraction media of the rapamycin loaded samples displayed lower cell viability than the Fe\textsubscript{2}O\textsubscript{3} nanotube group, indicating the possible inhibitory effects of rapamycin on the cell proliferation. Compared with the Rapa, PLGA and PLGA-Rapa had lower inhibition effects on the EC and VSMC growth, and this was due to a lower rapamycin concentration in the extraction media of PLGA and PLGA-Rapa. Besides, compared with PLGA, the PLGA-Rapa sample had stronger inhibitive effects on cell viability because of the presence of rapamycin in the PLGA coating. Comparing the proliferation of ECs and VSMCs, for all the samples after incubating for 1, 3 and 5 d, the viability of ECs was much higher than that of VSMCs, especially for the rapamycin loaded sample after being cultured for 3 and 5 d, with the viability of 49.21 \pm 2.61\% and 41.48 \pm 1.30\% for ECs and 18.49 \pm 0.76\% and 13.23 \pm 3.86\% for VSMCs, respectively, suggesting that the rapamycin had a better inhibitory effect on VSMCs.

During the early culturing time, although the PLGA and PLGA-Rapa covered samples did not have an excellent inhibitory effect on VSMC growth as the rapamycin loaded sample, a better long-term effect was expected for the sustained release of the rapamycin from the PLGA and PLGA-Rapa samples.\textsuperscript{40,41} When the samples were placed in the flowing-fluid environment (e.g. bloodstream), the drug therein would be released at a high eluting rate at the initial stage. Therefore, it is necessary to prepare a barrier coating on the drug loaded samples to obtain a sustained release and a locally high concentration of the rapamycin to inhibit the proliferation of VSMCs. Besides, incorporating the rapamycin into the cover film endowed the PLGA-Rapa with a better inhibitory effect on cell growth than PLGA. In general, the PLGA-Rapa sample would have a better effect on inhibiting the neointimal hyperplasia.

Immunofluorescence staining was carried out to investigate the cell adhesion and proliferation behavior. As indicated in Fig. 8(c–j), the number of endothelial cells on the different samples had no statistical differences. ECs had a good cytoskeleton and the microfilaments were clearly observed, which indicated a good function of ECs. After 5 days, as shown in Fig. 8(e), the Fe\textsubscript{2}O\textsubscript{3} nanotubes had the highest cell concentration density, and the Rapa sample had the lowest cell concentration density, suggesting that the activity of rapamycin could inhibit the proliferation of the EC. Compared with the PLGA-Rapa sample, more ECs were observed on the PLGA which was due to the rapamycin within the PLGA-Rapa film. As for VSMCs after incubation for 12 h, well-spreading cell morphologies could be observed on the Fe\textsubscript{2}O\textsubscript{3} nanotube sample; by contrast, VSMCs adhered to Rapa, PLGA and PLGA-Rapa displayed folded or narrow spreading morphologies. After 5 days, the Fe\textsubscript{2}O\textsubscript{3} nanotube sample was almost fully covered with VSMCs, while fewer VSMCs were observed in the other three media containing rapamycin. As shown in Fig. 8(j1), the morphology of smooth muscle cells was spindle, while in Fig. 8(j2–j4) morphological changes were observed for some VSMCs. All the results indicated that the rapamycin could effectively inhibit the proliferation of VSMCs and affect their cellular function. In Poon M's research, they proved that rapamycin not only inhibited cell proliferation but also inhibited VSMC migration.\textsuperscript{42}

According to the results of the cell proliferation assay and the immunofluorescent staining, the samples loaded with rapamycin could inhibit the proliferation of the two kinds of
cells, with a stronger inhibition effect on VSMC growth and migration, which was very important to reduce restenosis.

3.5 In vitro hemocompatibility evaluation of the samples

Hemolysis is regarded as an especially significant screening test index; a high plasma hemoglobin level normally indicates hemolysis and poor hemocompatibility of the materials. The hemolysis percentage of nanotubes (from 3.46 ± 0.61% to 3.99 ± 0.53%) was slightly increased compared to the untreated Fe (2.5 ± 0.58%) as shown in Fig. 9(a), and no statistical differences among the Fe₂O₃ nanotube samples with different diameters were observed. This value decreased to 1.96 ± 0.43% after being loaded with rapamycin; for the PLGA and PLGA-Rapa covered samples the hemolysis percentages were 1.41 ± 0.51% and 0.98 ± 0.33%, respectively. The hemolysis rates of all the samples were in the acceptable range according to the ISO 10993-4 standard (lower than 5%), suggesting that the samples would not lead to severe hemolysis when they came into contact with blood. Usually, when the materials and devices, for cardiovascular applications, were in contact with the blood, a low hemolysis was expected to avoid the breakdown of the erythrocytes and the formation of thrombus.

The PT and APTT tests are used to further evaluate the activation of the coagulation factor. The blood coagulation cascade includes intrinsic pathway, extrinsic pathway, and common pathway. APTT and PT are mainly used to examine the intrinsic and common pathway. The larger the PT and APTT values are, the lower the bioactivity of coagulation factors is. It can be seen from Fig. 9(b) that the PT and APTT of the nanotubes and pristine iron were higher than those of the original blood plasma, which indicated that the nanotubes had low activation to coagulation. The PT and APTT of Rapa samples were higher than those of the negative control, which suggested that the samples had low activation to coagulation. After being covered with PLGA and PLGA-Rapa, the PT and APTT reduced, but still higher than the negative control. The results demonstrated that the samples would not activate the coagulation factors.

Fig. 9(c–i) show the SEM images of the platelet attached on the samples after incubation for 1 h in PRP. Only a few platelets could be observed on the surface of Fe₂O₃ nanotubes and Rapa samples, and the shape of adhered platelets maintained integrity without the formation of pseudopodia. After being covered with the PLGA and PLGA-Rapa, some of the platelets were activated and pseudopodia were observed, but the number of platelets did not increase. All these results suggested that the nanotube coated Rapa, PLGA and PLGA-Rapa samples exhibited very good hemocompatibility.

4. Discussion

4.1 The advantages of fabricating Fe₂O₃ nanotubes on Fe

The Fe₂O₃ nanotubes, obtained by electrochemical anodic oxidation, were first reported by Grimes and co-workers based on their expertise in the fabrication of highly ordered titania nanotube arrays. Recently, a few attempts have been made to fabricate nanostructured Fe₂O₃ by the electrochemical technique on the surface of pure iron and stainless for photocatalysis applications.

This electrochemically surface functionalization method possesses the following advantages:

(i) There are no limitations in the shape and the size of the treated devices when using the anodic oxidation method to coat the Fe substrate. Fe–Mn alloy biodegradable scaffolds can be fabricated using a novel inkjet 3-D printing technique, and this aqueous-based anodic approach shows great adaptability to these sophisticated implants.

(ii) The diameter and the length of the Fe₂O₃ nanotubes, as well as their distribution and density, could be easily tuned by adjusting the process parameters that are involved in the anodic oxidation, such as voltage, oxidation time, electrolyte temperature, water content and so on.

(iii) The corrosion rate of the iron substrate can also be well controlled by manipulating the above mentioned process parameters, which in turn may ameliorate or deteriorate the iron biocompatibility.

(iv) The prepared porous/nanotubular Fe₂O₃ can be doped with Si, Ti, Nb and Mo, which may endow the coatings with potential and beneficial biological functions.

(v) The resulting nanotubular coatings can be exploited as a secondary platform to fulfill novel performances. In our study, Fe₂O₃ nanotubes were successfully utilized as rapamycin-delivery depots for potential drug-eluting applications. These nanotube arrays displayed several superior properties as drug-containing coatings:

(1) compared with a flat surface, the increased surface area and capillarity of these nanostructures allowed more loading amounts of drugs;

(2) the drug-loading methods were relatively simple and straightforward, such as electrospraying, lyophilisation, dipping coating, or vacuum impregnation technique;
(3) another promising feature of these nanoporous coatings was the feasibility to precisely control the pore (nanotube) size, distribution and density, so that the drug loading amount and release rate could be easily tuned.\textsuperscript{24}

In light of the above merits, we chose a typical anodic oxidation electrolyte containing ethylene glycol and ammonium fluoride solution,\textsuperscript{12} systematically investigated the effects of temperature, applying voltage and water content on the formation of anodic iron oxide (shown in the ESI\textsuperscript{†}), and finally obtained Fe\textsubscript{3}O\textsubscript{4} nanotubes with the diameter of 30 nm, 50 nm and 70 nm on the surface of pure iron, respectively. Moreover, these Fe\textsubscript{3}O\textsubscript{4} nanotube modified samples were further loaded by the anti-proliferation drug rapamycin and coated by PLGA films through a simple spin-coating process to control their drug release rate. And to the best of our knowledge, there are no studies attempting to evaluate the compatibility of the iron oxide nanotube surfaces for biomedical implant applications. It was expected that the oxide nanotubes prepared by the anodization approach could have favorable cell functions similar to TiO\textsubscript{2} nanotubes in terms of cell adhesion, proliferation and migration.

The results of XPS shown in Fig. 4(a) confirmed that the fluoride contents in the nanotube layers were as high as 10.12 at\%. So all the as-anodized Fe\textsubscript{3}O\textsubscript{4} nanotubes should be heat-treated. After annealing, the contents of fluoride dropped greatly to less than 1.7 at\% and the amorphous nanotubes crystallized to α-Fe\textsubscript{2}O\textsubscript{3}, as evidenced by the results of XPS and XRD.

4.2 \textit{In vitro} corrosion behaviors of the anodic oxidized Fe

An ideal biodegradable stent was expected to provide temporary mechanical opening support to the narrowed arterial vessel (for 6–12 months), then degrade and eventually be eliminated from the patient (after 12–24 months) through physiological pathways.\textsuperscript{2} Most of the reported iron-based stents remained intact after 1 year.\textsuperscript{10} Hence, a faster degradation rate of iron stents is desired for clinical applications, and this objective has been achieved either by using iron-based alloys\textsuperscript{10,52–56} or electroforming.\textsuperscript{11,57}

The degradation rate has a significant influence on the stent mechanical integrity, and the released degradation products may cause benign or malignant effects around the implantation site. Therefore, in order to offer a guide for preparing iron stents with desired performance, the \textit{in vitro} corrosion behaviors of the anodic oxidized Fe were investigated by both the electrochemical method in Hank’s solution and the immersion test in the cell culture medium. According to the electrochemical analysis results, the Fe\textsubscript{3}O\textsubscript{4} nanotubes with larger diameters could result in a higher corrosion rate of the iron substrate, with a sort order of 70 nm > 30 nm > 50 nm > Fe. As shown in Fig. 6(a3), the corrosion current densities (\(I_{corr}\)) of all the nanotube samples (around 37–45 \(\mu\)A cm\(^{-2}\)) were about one order higher than those of the pristine Fe (4.97 \(\mu\)A cm\(^{-2}\)), and also much higher than those of novel Fe–X binary alloys (X = Mn, Co, Al, W, Sn, B, C and S) designed by our research group previously.\textsuperscript{10} The immersion test results in Fig. 6(b) also suggested that anodization of iron could accelerate its degradation rate, especially for 50 nm samples (released the highest amount of iron ions about 19 \(\mu\)g mL\(^{-1}\)). It was speculated that the discrepancy between the electrochemical analysis and the immersion test resulted from the poor mobility of the media in the nanotubes. Except for the corrosion rate, the corrosion mode also plays a decisive role in determining the mechanical integrity of the biodegradable stent, such as pitting corrosion, crevice corrosion, uniform corrosion and so on. The presence of chloride ions in the physiological environment may lead to severe localized corrosion in Fe-based stents and result in local failure.\textsuperscript{5,10} From Fig. 6(c), it is found that after the immersion test, the microstructures of the nanotubes were well maintained, suggesting the relatively uniform corrosion of the Fe\textsubscript{3}O\textsubscript{4} nanotube coated Fe in the cell culture medium. Besides, the released Fe ions might be precipitated as porous hydrated ferric oxide and deposited on the Fe\textsubscript{3}O\textsubscript{4} nanotubes, possibly causing the thickening of the nanotube walls. The potentiodynamic polarization test can provide a fast prediction of the corrosion rate for materials screening and further research studies will be conducted (such as the dynamic immersion test or \textit{in vivo} test) in our future studies to predict the long-term corrosion mode or degradation behaviors of the samples.\textsuperscript{58}

This study indicated that creating oxidized nano-textured coatings on iron could increase the degradation rate of the substrate, which could be attributed to the increase of the exposed specific area of nanotubes to the aqueous media, and the anodic dissolution of iron by the Fe\textsubscript{3}O\textsubscript{4}/Fe interfacial (between the Fe\textsubscript{3}O\textsubscript{4} nanotubes and the Fe substrate) micro-galvanic reactions.\textsuperscript{16} Cheng \textit{et al.} prepared biodegradable Fe–Fe\textsubscript{3}O\textsubscript{4} composites with a higher corrosion rate than that of the pure iron.\textsuperscript{59} However, according to the investigation by Moravej \textit{et al.}, when the as-electroformed pure Fe was immersed into Hanks’ solution, the degradation products precipitated on the substrate (composed mainly of iron and oxygen) resulted in the lowering of the corrosion rate of Fe\textsuperscript{1+} this mismatch with our finding might be partially attributed to the microstructural and chemical differences between their precipitated layers and our anodic oxidized coatings.

4.3 \textit{In vitro} cytocompatibility of the surface-functionalized Fe

4.3.1 Fe\textsubscript{3}O\textsubscript{4} nanotube coating. The biological performance of the cells on the nanotube coatings displayed a strong size-dependent behavior. This was firstly reported by Park; they showed that the TiO\textsubscript{2} nanotube with a diameter of about 15 nm could effectively promote the adhesion, proliferation, and differentiation of mesenchymal stem cells\textsuperscript{60} and endothelial cells,\textsuperscript{13} and the ones with diameters of about 100 nm were found to induce cell apoptosis. They suggested that a 15–20 nm spacing distance would allow or force clustering of integrins into the nearly closest packing, resulting in optimal integrin activation. Nanotubes with a diameter of 100 nm almost completely prevented integrin from clustering for the formation of focal adhesion complexes, resulting in dramatically reduced cell proliferation, migration, and differentiation, and finally inducing apoptosis. However, the results are in part conflicting to other groups, which showed enhanced cell behavior using the
anatase phase of nanotubes within 70–100 nm diameters in comparison to flat Ti surfaces.45,61 These conflicts may be due to the different surface topography, coating crystalline structures, and the use of different cell types.

Here, we showed that the 50 nm Fe$_2$O$_3$ nanotubes provided better cellular functioning than the flat iron, exhibiting better cell viabilities of endothelium but not for smooth muscles cells. The reasons for this may stem from three aspects, the chemical cues, the physical structures and the synergistic effects of both factors. (1) Fe (ions) is an essential element for life due to Fe-containing enzymes and proteins which are responsible for the transport, storage and activation of molecular oxygen, the decomposition of lipid, etc.62 Recent investigation indicates that the appropriate concentration of Fe ions can not only produce favorable effects on the metabolic activity of endothelium but also suppress the VSMC proliferation and migration.7,63 Compared with other samples, the 50 nm groups released the highest amount of iron ions (about 19 µg mL$^{-1}$) when being immersed in DMEM (as shown in Fig. 6b), which was much lower than the suggested inhibited concentration of 50 µg mL$^{-1}$ for human endothelial cells.63 (2) It has been reported that endothelial cells function better on periodic or patterned nanostructures opposed to random nanostructures, and the nanotube surface significantly enhances EC proliferation and decreases VSMC proliferation,43 which allows for enhanced cellular expansion and an overall increase in cell/substrate interaction and cell migration. The holes, edges, and ledges of 50 nm Fe$_2$O$_3$ nanotubes may provide more beneficial nano-cues and channels for ion, nutrient, and protein exchange,64 helping to form a more natural basal membrane (ECM) in endothelial hemostasis.65 (3) The specific reasons for the differences in cell viability and proliferation between 50 nm nanostructures and other groups are unclear. This may also originate from the synergistic effects of the chemical cues (the released Fe ions from 50 nm Fe$_2$O$_3$) and the physical structures (50 nm nanotube coatings) on cell behaviors.

Considering the biocompatibility of the Fe$_2$O$_3$ per se, both the in vivo65 and in vitro66 studies suggested that Fe$_2$O$_3$ is well tolerated and displays excellent biocompatibility, with widespread biomedical applications in drug delivery, cell imaging and labeling, magnetic hyperthermia and so on.66 Some studies suggested that iron oxide nanoparticles could be incorporated into lysosomal vesicles and degraded by lysosomal R-glucosidase.67,68 In the long term, the prepared Fe$_2$O$_3$ nanotube coating might break into nanoparticles and be transported to some organs (such as lungs, liver and kidneys) by blood circulation where they can be stored or cleared.69 PLGA is a widely investigated copolymer for being used as a coronary stent or a stent coating, and its degradation behavior is crucial for its usage in drug-eluting stent systems; it can produce acidic degradation products which in turn was expected to enhance the corrosion rate of the underlying substrate. Compared with pure Fe, PLGA displays a much faster degradation rate in vivo. When the PLGA coating was completely degraded, the residual Fe$_2$O$_3$ nanotubes were expected to promote the re-endothelialization of the samples. The detailed synergistic effects of PLGA with Fe$_2$O$_3$ nanotubes on the corrosion rate of Fe will be studied in our future work.

4.3.2 Fe$_2$O$_3$ nanotubes with rapamycin and PLGA. Fe stents are biodegradable, but may still bear the same potential complications that are induced by other bare metal stents, the delayed endothelialization of stents and the proliferation of vascular smooth muscle cells (VSMCs), resulting in the formation of thrombosis and in-stent restenosis, respectively. Therefore, anti-proliferative drugs should be incorporated onto/into the stents to tackle this problem.18 In our study, except for their roles in mediating the surface nanostructure and increasing the corrosion rate of the Fe substrate, for the first time, Fe$_2$O$_3$ nanotubes were employed as nano-depots for drug loading.

When used in the cardiovascular scenario, drug-containing stents were expected to have a sustained drug release time and an effective drug amount to promote endothelialization and avoid in-stent restenosis. Rapamycin (sirolimus)$^{70,71}$ and paclitaxel$^{72}$ are frequently exploited to make drug-eluting stents to reduce the in-stent restenosis. As shown in Fig. 7, due to its open-structure and high drug loading amount and concentration gradient, a severe burst release of rapamycin was observed. After being coated by PLGA, the stents showed a considerably extended overall drug release of 30 days, confirming the ability of this approach to achieve a long and sustained drug release, which may be required, for example, for effective inhibiting vascular cell hyperplasia. The inhibitory effects of rapamycin on the proliferation of cells were concentration and time dependent,39 and both ECs and VSMCs cultured in the extraction media of the rapamycin loaded samples displayed lower cell viability than the Fe$_2$O$_3$ nanotube group, especially for the VSMC. The cytological mechanism underlying the selective response of ECs and SMCs to rapamycin has not been fully elucidated, and many research studies have been performed to evaluate its effect on vascular cells. Studies by Matter et al.$^{73}$ suggested that rapamycin showed stronger inhibiting effects on VSMC proliferation than tacrolimus and tacrolimus exerted less antiproliferative or anti-migratory effects on ECs compared with rapamycin. Rapamycin can upregulate the cyclin-dependent kinase inhibitor p27$^{kip1}$ in VSMCs, resulting in cell-cycle arrest at the G1 to S transition. Besides, rapamycin also inhibits other important cellular functions, including protein translation.$^{74,75}$ This could be a possible explanation on why rapamycin displayed a stronger inhibitory effect on VSMCs than ECs. And these selective responses might also be ascribed to the released Fe ions, which could produce favorable effects on the proliferation of endothelium and suppress the VSMC proliferation.7,62 Zhu et al.$^{70}$ prepared a three-layer hybrid coating on the Mg stent, with dextran/rapamycin as the first and third layers and polyglutamic acid as the second layer; this coating design concept can also be applied to the surface-functionalization of Fe$_2$O$_3$ nanotubes, to endow the coatings with effective anti-inflammatory and anti-proliferative properties.

4.4 In vitro hemocompatibility of the coated Fe

The choice of stent materials, site of implantation and blood flow will have a primary influence on the degree of thrombus
formation within the stent.\textsuperscript{76} Hence it is important that the stent materials or the stent coatings are hemocompatible.

According to the results of the blood biocompatible evaluation tests, although the hemolysis rate of Fe\textsubscript{3}O\textsubscript{4} nanotubes was slightly higher than that of pristine iron, it is lower than that of 316L stainless steel (5.03) as reported,\textsuperscript{77} and in the acceptable range according to the ISO 10993-4 standard. In addition, no obvious differences in the platelet adhesion on the anodized and pure iron plates were observed, in terms of platelet morphologies and amounts. Previous studies demonstrated that the number of platelets on the pristine iron was significantly less than that on 316L stainless steel and Mg-Mn-Zn alloy.\textsuperscript{78} This suggests that our anodized nanotube iron exhibits very good hemocompatibility.

5. Conclusions

In our present study, for the first time, we have successfully fabricated an endothelial favorable and thromboresistant rapamycin-loaded nanotubular $\alpha$-Fe\textsubscript{2}O\textsubscript{3} coating on Fe for biodegradable drug-eluting stent applications. The samples anodized at 30 V for 15 min (the 50 nm-Fe\textsubscript{2}O\textsubscript{3} nanotube arrays) displayed a faster corrosion rate than the pristine Fe, which were further subjected to drug loading by dipping and PLGA coating by spin-coating. The static immersion test indicated that PLGA and PLGA-Rapa effectively reduced the initial burst release and showed a considerably extended overall drug release of 30 days, confirming the capability of this approach to achieve a long and sustained drug release. The endothelial cells (ECs) on the coated samples showed higher cell viability than the vascular smooth muscle cells (VSMCs), with possible outcomes to promote re-endothelialization and decrease VSMC proliferation. In addition, the samples exhibited very good hemocompatibility. Therefore, the Fe\textsubscript{2}O\textsubscript{3} nanotube modified iron, combined with its biodegradable property and drug loading capability, is probably a promising candidate for the next generation of vascular materials, and in vivo studies should be conducted to test the feasibility of translating this nanostructuring approach to iron stents.

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Notes and references
