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Different complex surfaces of polyethyleneglycol (PEG) and REDV ligand to enhance the endothelial cells selectivity over smooth muscle cells

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ABSTRACT

Arg-Glu-Asp-Val (REDV) peptide with endothelial cells (ECs) selectivity was immobilized onto PEG based polymeric coating via the active *p*-nitrophenyloxycarbonyl group. The adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) onto surface modified either by REDV end-tethered polyethylene glycol (PEG) or by the complex of free PEG and REDV were investigated to understand the synergic action of nonspecific resistance of PEG and specific recognitions of REDV. Cell culture results indicated that the surfaces end tethered by REDV peptide via PEG "spacer" ($n = 1$, 6, 10) exhibited slight EC selectivity and showed small difference between different lengths of PEG chain. Both separate-culture and co-culture of HUVECs and HASMCs indicated that the introducing of free PEG into REDV tethered surface inhibited HASMCs adhesion significantly and remained a high level of HUVECs growth. Furthermore, the surface with short free PEG chain ($n = 6$) was much more effective to enhance ECs selectivity than long EG chain ($n = 23$). The combination of nonspecific resistance of short free PEG and the ECs selectivity of REDV peptide presents much better ability to enhance the competitive adhesion of HUVECs over HASMCs.

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1. Introduction

Thrombogenicity and intimal hyperplasia have been the most common causes of cardiovascular implantation [1–3]. Previous research have demonstrated that exposed intimal or luminal area of implantation may lead to thrombosis by platelet deposition, while injury induced the migration of smooth muscle cells (SMCs) and the delay of re-endothelialization are the major pathophysiological causes which lead to neointima formation [4,5]. Vessel endothelium plays an important role in keeping the integrity of vessel, preventing thrombosis and hyperplasia. The formation of an endothelial cell monolayer on the luminal surface of vascular implantation helps to resolve the problem of thrombogenicity and intimal hyperplasia [6,7]. As a result, re-endothelialization has been proven to provide an available technique to prevent in-stent restenosis (ISR) and late stent thrombosis (LAST) [8].

Comparing with endothelial cells (ECs) pro-seeding method, in situ endothelialization of material after implantation inside the body provides a fascinating alternative strategy for the rapid re-endothelialization of cardiovascular implantation [9,10]. Lots of studies have proved that extracellular matrix (ECM) molecules or the synthetic cell adhesive peptides such as Arg-Gly-Asp (RGD) enhance ECs' adhesion and proliferation onto surface [11–13]. However, very little attention has been paid to SMCs. Due to the complex competition of different cells *in vivo*, the selective promotion and competition of ECs with other cells such as SMCs [14–16] play important roles in achieving a successful in situ endothelialization process. Recent researchers including ourselves [4,17,18] have demonstrated that the immobilization of ECs specific ligand onto a biocompatible surface could enhance the ECs selectivity over SMCs and lead to a rapid in situ endothelialization of materials. Accordingly, the exploration of other possible approach to enhance ECs selectivity over SMCs is interesting to achieve rapid in situ endothelialization on material surface.

The external region of cell membrane, known as the glycocalyx, is dominated by glycosylated molecules, which directs specific interaction such as cell–cell recognition and contributes to the steric repulsion that prevents undesirable non-specific adhesion of other molecules and cells [19]. The densely packed, highly hydrated polysaccharide provides a physical basis for maximizing entropic repulsion and prevents non-specific adhesion. Desirable adhesive interaction and biological specificity are often achieved through the specific interaction between cell and surface glycoprotein receptor molecules [20–22]. In a word, the combination of non-specific resistance and specific interaction provides a fascinating approach to achieve high selectivity in the complex *in vivo* system. Following this biological perspective, polyethylene glycol (PEG) based copolymers have been explored to construct glycocalyx-mimic surfaces. PEG chain has been regarded as the most effective structure to reduce non-specific interaction [23–25] and it could be

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end-tethered ligand to obtain specific cell–substrate interaction [26–29]. Nevertheless, very little attention has been paid to cell-specific interaction of random PEG copolymers combined free PEG with specific ligand. The peptide sequence Arg-Glu-Asp-Val (REDV) is specifically recognized by the integrin α4β1 which is abundant on ECs and scarce on SMCs [30]. REDV-coated surfaces have been proved to mediate ECs attachment and migration without SMCs adhesion [31,32]. We hypothesize here that the combination of nonspecific resistance of PEG and endothelial cell preferential ligand will enhance the competitive adhesion of ECs over SMCs, which may be further explored as an available method for in situ endothelialization.

In this research, a series of reactive copolymer were prepared by conventional radical polymerization of butyl methacrylate (BMA), polyethylene glycol methacrylate (PEGMA) and *p*-nitrophenyloxycarbonyl poly (ethylene glycol) methacrylate (MEONP) with different oxyethylene chain lengths. REDV peptide was immobilized onto the reactive polymeric coating via the active *p*-nitrophenyloxycarbonyl group. The adhesion and proliferation of HUVECs and HASMCs onto surfaces modified either by REDV end-tethered PEG or by the complex of free PEG and REDV were examined to investigate the synergic effect of nonspecific resistance of PEG and specific recognition of REDV.

2. Materials and methods

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA) was purchased from Sigma–Aldrich (China) and purified by distillation under reduced pressure. Poly (ethylene glycol) methacrylate (Mn = 360 (n = 6, n means the repeating units of EG) and 526 (n = 10)) and poly (ethylene glycol) methyl ether methacrylate (Mn = 1100 (n = 10)) were commercially obtained from Aldrich Chemical Co. and used without further purification. Butyl methacrylate was purified by distillation under reduced pressure (LingFeng of the Shanghai Chemical Reagent Co., Ltd.). *p*-Nitrophenylchloroformate was purchased from DaRui Chemical Co., Shanghai. α,α′-Azobisisobutyronitrile (AIBN) was recrystalized in methanol solvent. Arg-Glu-Asp-Val (REDV) peptide was obtained from Shanghai Science Peptide Biological Technology Co., Ltd. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and fluorescein diacetate (FDA) were obtained from Sigma–Aldrich, China. Orange [5-(and 6)-(((4-chloro-methyl)-benzoyl)-amino)-tetramethyl-rhodamine, CMTMR] cell tracker dyes was purchased from Molecular Probes. HUVECs were friendly provided by Second Hospital Affiliated to Medical College of Zhejiang University and HASMCs were purchased from Beifangweiye development Co., Ltd. (Shanghai, China).

2.2. Synthesis of MEONP and copolymers

As one of monomer units, MEONP was synthesized as previously described [33,34]. Briefly, into a 100 ml four-necked round-bottomed flask equipped with a dropping funnel, a thermometer, and a magnetic stirrer, 0.03 mol of HEMA/PEGMA (Mn = 360, 526) and TEA (4.2 ml) were placed, then 20 ml of chloroform was added to the mixture. After the solution was cooled at −20 °C, *p*-nitrophenylchloroformate (6.05 g) dissolved in 15 ml of chloroform was added drop by drop to the stirred solution over a period of 1 h. The temperature of the reaction mixture was maintained at −20 °C for 4 h. The precipitate formed in the reaction mixture was triethylamine hydrochloride (TEAC), which was then filtered off. The solvent in the filtrate was evaporated under reduced pressure. A small amount of dry diethyl ether was added the residue to remove the dissolved TEAC, followed by filtration. By evaporating the filtrate under reduced pressure, MEONP was obtained as a yellow oily liquid. We marked as MEONP-*n*1 (HEMA), MEONP-*n*6 and MEONP-*n*10 (PEGMA). The structure of MEONP was confirmed by ^{1}H NMR (500 MHz, Avance DMX500, Bruker).

$P(EG)_n$BN was synthesized by conventional radical polymerization of PEGMA (Mn = 360, 1100), BMA, MEONP-*n*1 (MEONP-*n*6) using AIBN as an initiator. Polymerization was carried out in polymerization tube at 60 °C about 24 h. After polymerization, the reaction mixture was precipitated using an excess amount of dry diethyl ether, repeated twice. BN-n10 was synthesized using BMA, MEONP-*n*10 with the same method. The ratio of monomer unit composition in polymer was determined by ^{1}H NMR and UV spectrum at 275 nm (UV-VIS-NIR Spectrophotometer (Bio), Cary 100 Bio).

2.3. Conjugation and immobilization of REDV peptide

Polyethylene terephthalate (PET) film was used as substrate for polymer coating. After polymer was completely dissolved in tetrahydrofuran (THF), the process of dip-coating was performed. The substrate was vertically dipped into the solution and then taken out with constant speed, repeated six times. The membrane was dried under vacuum for 24 h.

After that, the substrates were put into a 24-well plate and then REDV solution (phosphate buffer solution pH 7.4, 200 μg/ml) was added to the disks at 4 °C for 24 h incubation. Then, the substrates were rinsed three times with PBS. To eliminate the negative effect of phenyl group, BSA as a blocking reagent (in PBS solution, pH 7.4, 200 mg/ml) was added into separate wells to block the areas unoccupied by REDV peptide [35]. The sample was rinsed with PBS five times again and then placed into vacuum oven overnight.

2.4. Surface characterization of the developed materials

The MEONP unit contains active ester groups for the conjugation of peptide via the oxyethylene chain. As a result, the total amount of REDV onto polymer surface could be analyzed by calculating the amount of *p*-nitrophenol as a leaving group in reaction solution [33,36]. UV–vis spectrum (UV-VIS-NIR Spectrophotometer (Bio), Cary 100 Bio, 405 nm) was measured to investigate the absorbance of *p*-nitrophenol in aqueous solution.

Contact angle measurements were carried out on a KRUSS DSA 10-MK2 goniometer on sheet-samples employing the sessile-drop and captive bubble measuring methods. For sessile-drop method, a water drop was dispensed onto the substrate investigated. In the captive bubble technique, the solid sample was placed in water surface with glass sheet covered. A small air bubble made by microsyringe was attached to the solid surface. Then the contact angle was measured. Five different positions were measured to get the average contact angle.

Spectroscopic ellipsometry was used to investigate the stability of polymer coating. In detail, the polymer film was fabricated as solvent evaporation and nitrogen drying processes on silicon slices. The thickness of polymer coating was determined by ellipsometry, and then the film was incubated in PBS. After three days incubation, the polymer coating was picked out, dried by nitrogen stream. The thickness of the polymer coating was measured again. For each modified film, spots from three different silicon wafers were measured to gain the average and standard deviation of the thickness. The percent of the remaining polymer films thickness with the time was defined as the film stability.

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