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Synthesis of Polypyridine-graft-PEG Copolymer for Long-term Stability of Nonfouling Character

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Polypyridine grafted with poly(ethyrene glycol) (Py-g-PEG) have been synthesized. Radical copolymerization of methyl-terminated PEG macromonomer with 4-pyridylmethyl methacrylate homogeneously proceeded and the obtained copolymer spontaneously adsorbs from aqueous solution onto gold surfaces, where the pyridine parts act as the multipoint anchor to the surface and the PEG parts provide the strong steric repulsion between the chains. As a result, the highly protein repellent and stable surface was constructed through multipoint pyridine attachment as compared with singlepoint pyridine attachment. Py-g-PEGs thus shynthesized are promising material to functionalize metal and semiconductor material and to self-assemble into micelle in biotechnological and biomedical field.Instructions for the preparation of camera-ready manuscripts for publication are given.

Key words: multipoint anchor, pyridine, interfacial stabilization, nonfouling

1. INTRODUCTION

Nonspecific fouling of the device surface with biocomponents including plasma proteins is one of the crucial issues in biotechnological and biomedical applications. Biofouling usually starts with the nonspecific adsorption of proteins, and this, in turns, triggers the deposition of biological cells, bacteria, or other micro-organisms. These biofouling may cause serious problems such as blood coagulation and immune responses on medical device surfaces. To prevent nonspecific biofouling, a variety of modifications on the surface has been carried out[1-4]. Modification by poly(ethylene glycol) (PEG) tethered chains leads to a significant reduction in the nonspecific interaction of biological molecules with the surface because PEG is highly hydrophilic and has appreciable chain flexibility inducing an effective exclusion volume effect in an aqueous environment.

Since Nuzzo and Allara reported the first preparation of organthiol monolayer assemblies on a gold surface in 1983[5], various studies[6-11] of sulfur containing PEG at the chain end, which adsorbs onto gold surfaces, has been reported for biomedical applications such as highly sensitive diagnosis and biosensor and for obtaining basic insights into the biocomponents-surface interactions[12-15]. However, one concern with using monomeric mercapto-ended PEGs to modify gold surface is possible inadequate stability when exposed to buffer with nonphysiological pH, oxidative chemicals, electrochemical environments, or large biomolecules (thermal deposition of PEG). In this study, we focused on the gold-pyridine binding, which is known to be highly stable against enzymatic oxidation and also have high affinity to many metals[16-19]. Furthermore, it is now generally accepted that the Au-adsorbate bond formed on gold surfaces plays a key role in governing the structure and stability of the monolayers. The increase in the number of bonding sites on gold surface per adsorbate molecule should result in the increased stability of the adlayers. Consequently, the formulation of polymeric surfactants of block or grafted coplymers have the main advantages of their strong adsorption at the solid/liquid interface, where the B part of the polymer acts as the multipoint anchor to the surface, and the A part provide the strong steric repulsion between the chains. To achieve both protein repellent effect by PEG and interfacial stability through multipoint anchoring by pyridine moieties, a series of chemisorbing polymers have been synthesized; The polypyridine-graft-PEG polymer (Py-g-PEG) has pyridine anchoring side chains and methyl-terminated poly(ethylene glycol) (PEG) side chains grafted to polymer backbone. The plural pyridine side chains provide multipoint attachment to the gold surface, while the PEG side chain should reduce nonspecific fouling of proteins. In this report, synthesized Py-g-PEG copolymers were immobilized on gold surface, and amount of protein adsorption was evaluated by flowing of BSA on each surfaces using SPR instrument. Since the required performance of the Py-g-PEG polymer depends not only upon its adsorption density but also on its conformation and orientation at the interface, one of the main objectives in this study is to investigate the effect of the variation in PEG grafting density on protein adsorption and interfacial stability. For this purpose, numerous Py-g-PEG with different PEG/Py unit ratios were synthesized.

2. EXPERIMENTAL PART

2.1 Materials.

Commercial methacrylic acid (Wako), 4-pyridinemethanol (TCI),

N,N'-dicycrohexylcarbodiimide (Wako), 4-(1-pyrrolidinyl)pyridine (Wako), SUNBRIGHT ME-020AS (a gift from NOF Corporation), and 2-2'-azobis-(isobutyronitrile) (Wako) were used as received. Bovine serum albumin were purchased from Sigma Chemical Co. The α-methyl-ω-methacryloyl-PEG (Mn = 2080) was purified with following procedure: 50 wt. % solution in water (Aldrich) was dried over under reduced pressure, with dry MgSO4. The resulting amorphous PEG was dissolved in THF followed by addition to cold 2-propanol, and the obtained precipitate was freeze-dried from benzene, yielding white powder. Reaction solvents were purchased from Wako dehydrated grade. All water used was purified by treating in reverse osmosis unit followed by a Millipore unit (18mΩ resistivity).

2.2 Preparation of 4-Pyridylmethyl-methacrylate.

To synthesize polypyridine-graft-PEG copolymer, 4-pyridilmethyl-methacrylate as a pyridine monomer was synthesized. Methacryl acid (4.73 g, 55 mmol), 4-pyridine methanol (5.45 g, 50 mmol), and 4-(1-pyrrolidinyl)pyridine (740 mg, 5 mmol) were dissolved in dry dichloromethane (100 ml) in a glass vessel. After N,N'-dicyclohexylcarbosiimide (11.3 g, 55 mmol) was added to the solution, the reaction mixture was stirred for 1 h at r.oom temperature. After the resulting insoluble urea was removed with filtration, the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, hexane / ethyl acetate) yielding colorless oil (8.1 g, 46 mmol, Y. = 91 %).

2.3 Copolymerization of Methyl-Terminated PEG Macromonomers with 4-Pyridylmethyl Methacrylates.

A series of Py-g-PEG were newly synthesized with radical copolymerization. As shown in scheme 1, 4-pyridylmethyl-methacrylate (177 mg, 1.0 mmol), several amount of α-methyl-ω-methacryloyl-PEG (Mn = 2080), and AIBN (1 mol% of monomer) were dissolved in dry DMF (ca. 10 times amount of monomer mass). After the mixture was frozen and degassed 3 times, the solution was stirred for 24 h at 60°C. The reaction mixture was dropped into 2-propanol (100 ml), the solution was stirred for few minutes. The resulting precipitate was separated with centrifugation and freeze-dried from benzene to give white powder.

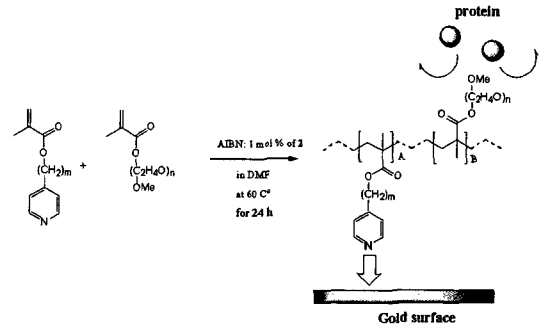
2.4 Preparation of α- methyl-ω-methyl(4-pyridilmethyl-carboxylate)-PEG, (single-Py-PEG).

Synthetic route of pyridine-PEG (single-Py-PEG) was shown in scheme 2. After α-methyl-ω-methyl(N-hydroxysuccinimidyl-carboxylate)-PEG (SUNBRIGHT ME-020AS) (1.14 g, 0.50 mmol), 4-pyridinemethanol (109 mg, 1.0 mmol), N,N'-dicyclohexylcarbosiimide (113 mg, 0.55 mmol), and 4-(1-pyrrolidinyl)pyridine (15 mg, 0.10 mmol) were added to dry dichloromethane, the mixture was stirred for .24 h at room temperature. After the resulting insoluble urea was removed with filtration, the solvent was removed under reduced pressure. The residue was precipitated from 2-propanol at 4°C, the precipitate was separated with centrifugation, freeze-dried from benzene to yield white powder (697 mg).

2.5 Analysis.

1H NMR spectra were obtained using CDCl3 solution with JEOL AL-300 spectrometer at 300 MHz. Tetramethylsilane was used as an internal standard. The molecular weight and molecular weight distribution of the synthesized PEG were obtained using TOSOH HLC8220 GPC equipped with a gel permeation column (TSKgel G4000HR + G3000HR). DMF containing10 mM LiCl was used as an eluent. Surface plasmon resonance (SPR) instrument (Biacore X; Biacore AB, Uppsala, Sweden) was used to investigate surface modification and protein adsorption.

3. RESULTS AND DISCUSSION



Scheme 1. Synthetic route of polypyridine-grafted-poly(ethylene) glycol copolymer.

Radical copolymerization of methyl-terminated PEG macromonomer with 4-pyridylmethyl methacrylate shown in Scheme1 homogeneously proceeded. Thus, polypyridine-graft-poly(ethylene glycol) copolymers (Figure 1) with different copolymer architectures in terms of PEG grafting ratio were successfully

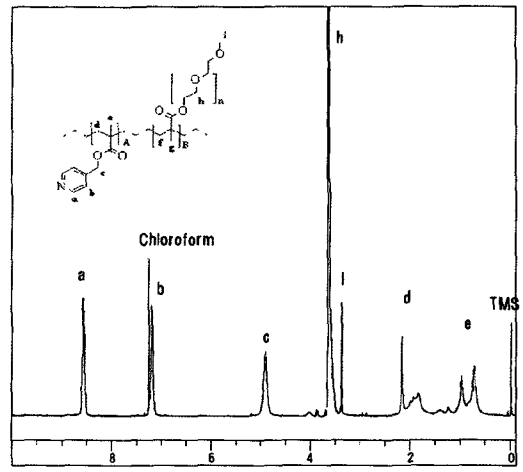


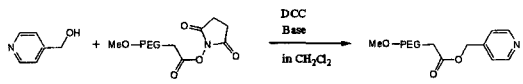
Figure1. ^{1}H NMR spectrum of typical Py-g-PEG (PEG/Py=0.08) in $CDCl_3$ at room temperature.

synthesized and quantitatively characterized by 1H NMR. As can be seen in the most typical ^{1}H NMR spectrum within the obtained Py-g-PEG, it is clear that each protons is assigned to obtained graft copolymer,

Table1. Chemical properties of synthesized Py-g-PEG.
* calculated from Mn, and ^{1}H NMR integral ratio of protons derived from pyridine (a) and PEG (h) in Figure1.

m	n	Additive of PEG monomer (mol%)	PEG/Py ratio	Mn	Mw/Mn	PEG+Py Unit No	PEG Unit No
1	43	3	0.03	40027	1.46	175	4.7
1	43	10	0.08	51500	1.36	159	12.2
1	43	40	0.28	34061	1.28	57	12.6
1	43	60	0.56	58864	1.29	69	24.5

where the assignments were carried out using pyridine, MMA, and PEG and are described in the figure. The number of each pyridine and PEG molecules per obtained graft copolymer can be controlled by changing the initial ratio of PEG/Py, resulting in the preparation of four Py-g-PEG copolymer samples with systematically varying grafting raios as summarized in Table 1 and Figure 2. A systematic notation is used for the stoichiometry of Py-g-PEG polymers, indicating the average molecular weight of PEG and the grafting ratio. The grafting ratio is expressed as the number of PEG side chains divided by the numer of pyridine side chains (PEG/Py ratio). For example, a polymer labeled Py-g(0.28)-PEG(2) has PEG side chains of molecular weight 2 kDa (43 EO-mer), and a grafting ratio of 0.28 (0.28 PEG chains per pyridine unit). Each PEG/Py ratio in Table 1 was calculated from Mn, and 1H NMR, taking ratios of the areas derived from pyridine (a) and PEG (h) in Figure 1. Calculated PEG/Py ratio was increased according to increase of added PEG macromonomer, thus, four Py-g-PEG copolymer



Scheme 2. Synthetic route of poly(ethylene)glycol having pyridine group at the chain end.

samples, PEG/Py=0.03, 0.08, 0.28, 0.56, has been synthesized. A series of Py-g-PEG was provided to surface modification studies as a sample of multipoint attachment of pyridine molecules on gold surfaces. To compare the interfacial stability of pyridine between the multipoint attachment and singlepoint attachment, PEG having pyridine group at the end of the chain was synthesized. Successive synthesis of single-Py-PEG shown in Scheme 2 was also identified from ^{1}H NMR spectra using $CDCl_3$ as a solvent with tetramethylsilane as an internal standard. The substitution ratio calculated from peak area of ^{1}H NMR spectrum was ca. 70 %. Single-Py-PEG thus synthesized was provided to surface modification studies as a sample of singlepoint attachment of pyridine molecules on gold surfaces.

Immobilization of a series of Py-g-PEG or single-Py-PEG on the gold sensor chip surface was performed using surface plasmon resonance (SPR) instrument. 1 mM HCl containing 1M NaCl of Py-g-PEG or single-Py-PEG solutions were prepared in the concentration of 0.1 mg/mL and injected at a flow rate of 10 μL/min for 10 min at 37°C under running of degassed deionized water. In order to increase (or modulate) the immobilized amount of PEG, the process of PEG injection was repeated several times according to the previous paper[20]. In consequence, 100 μL of Py-g-PEG or single-Py-PEG was immobilized on gold sensor chip by triple repetitive injections. To estimate the stability of PEGylated surfaces, BSA (1 mg/mL) adsorption study was performed on each PEGylated surfaces by SPR. To estimate the stability, adsorbed amount of BSA measured immediately after PEG immobilization was compared with that after 1 day and 2 week PEG modification. At first, immediate adsorption was measured by flowing 100 μL of BSA aqueous solution on each PEGylated surface and bare gold surface (Figure 2a). On all PEGylated surfaces, immediate BSA adsorption clearly decreased, while on a bare gold surface BSA was significantly adsorbed, Especially on Py-g-PEG surfaces, BSA adsorption was appreciably inhibited compared with single-Py-PEG surface. It is further noted that on Py-g-PEG surface, BSA adsorption was decreased with increase of PEG/Py ratio. On single-Py-PEG surface, amount of BSA adsorption was about 2 times more than that of Py-g-PEG surfaces. This difference may be due to the density and stability of the PEG chain, relating to the number of pyridine group existed in 1 molecule. Single pyridine located at the end of PEG chain can be

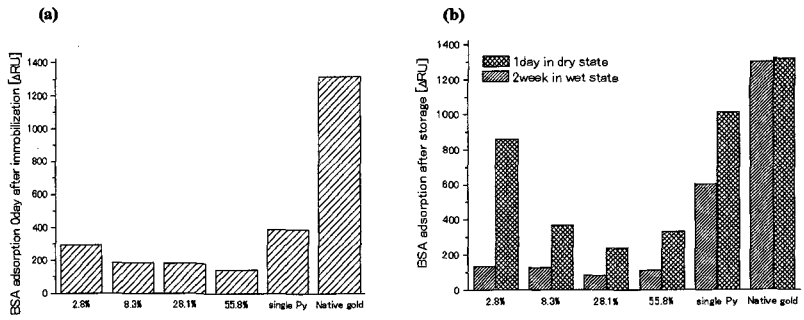


Figure2. BSA adsorption on single- and Py-g-PEG surface and bare gold surface; immediate adsorption after immobilization (a), 1day after incubation in dry state (b-right), and 2week after incubation in wet state (b-left).

considered to have weak affinity with gold surface, immobilized single-Py-PEG may be partly exchanged by BSA adsorption through thermal desorption of single-Py-PEG. Then, BSA adsorption 1day after PEG immobilization was measured by flowing same amount of BSA aqueous solution on maintained PEGylated surfaces in dry state at 37 °C for 1 day (Figure 2b). BSA adsorptions were drastically changed as shown in Figure4-b. Although the amount of BSA adsorbed were significantly increased on all PEGylated surface as compared with immediate adsorption, single-Py-PEG surface showed the worst surface stability, where the adsorption was almost the same as that of bare gold surface. On the other hand, Py-g-PEG surface showed still lower BSA adsorption, depending on the copolymer architectures. As the PEG/Py ratio was increased, the amount of BSA adsorbed on the surface significantly decreased up to a PEG/Py ratio of 0.28. It is interesting to note that a further increase in PEG/Py ratio (=0.55) resulted in a slight increase in BSA adsorption. Although Py-g(0.55)-PEG(2) surface showed the best effect to inhibit BSA adsorption measured immediately after PEG immobilization, when maintained for 1day in dry state, the inhibition effect of BSA adsorption disappeared in some extent and was almost the same as Py-g(0.08)-PEG(2) surface This result is due to the interfacial stability of the copolymer which will be caused by pyridine content existed in a Py-g-PEG copolymer. Scarce pyridine content may causes weak affinity with gold, resulting in exchange adsorption to BSA. However, Py-g(0.56)-PEG(2) surface still remained lower adsorption than that of single-Py-PEG surface. These result suggested that pyridine multipoint attachment achieved to construct more stable PEG surface compared with single attachment.

In the consideration of application of this copolymer as a biomaterial, stability in wet state at the temperature of 37 °C is more important. Thus, BSA adsorption test was performed in wet state. Py-g-PEG or single-Py-PEG immobilized SPR gold sensor chip was stored in deionized water at 37 °C for 2 weeks. Then BSA adsorption was measured by SPR instrument in the same way as dry state. As shown in Figure 3b, all PEGylated surfaces inhibited BSA adsorption compared with dry state. It should be noted that adsorbed amount on Py-g(0.56)-PEG(2) surface was higher than Py-g(0.28)-PEG(2) surface in wet state as well as dry state. In addition, as the PEG/Py ratio was increased, the amount of BSA adsorbed on the surface significantly decreased up to a PEG/Py ratio of 0.28. These trends were completely same as dry state, and furthermore, BSA adsorption was more inhibited in wet state compared with dry state. For the biomaterial application, obtained interfacial stability in wet state is central topic for long-term utility, suggesting that optimization of PEG/Py ratio is highly important. Shown above, Py-g(0.56)-PEG(2) surface most inhibited immediate BSA adsorption in all PEGylated surfaces. On the other hand, 1day (in dry state) or 2 week (in wet state) after immobilization, Py-g(0.28)-PEG surface most inhibited BSA adsorption in all PEGylated surfaces. Immediate BSA adsorption after the copolymer immobilization reflects PEG density on the surface, however, BSA adsorption after 1 day or 2 week immobilization reflects surface stability, intimately related to pyridine content. The most optimized polypyridine-graft-PEG polymer (Py-g-PEG) at PEG/Py=0.28 was obtained to modify the gold surface with both suitable nonfouling and interfacial stability.

4. CONCLUSION

The highly protein repellent and stable surface was constructed by using newly synthesized polypyridine-graft-PEG copolymer. Multipoint pyridine attachment sites achieved more stable surfaces compared with singlepoint pyridine attachment. PEG/Py=0.28 of Py-g-PEG constructed the most protein repellent and stable surface, suggesting that the PEG/Py ratio was critical for both nonfouling and surface stability. Polypyridine-g-PEGs thus shynthesized are promising material in biotechnological and biomedical field, which may have high utility to functionalize metal and semiconductor material and to self-assemble into micellization (hydrophobic of pyridine unit and hydrophilic of PEG unit) for drug and gene delivery.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

1. C. G. P. H. Schroen, M. A. Cohen Stuart, K. van der Voort Maarschalk, A. van der Padt, K. van't Riet, Langmuir 11, 3068 (1995).
2. S. J. Sofia, V. Premmnath, E. W. Merrill, Macromolecules 31, 5059 (1998).
3. W. Norde, D. Gage, Langmuir 20, 4162 (2004).
4. A. Roosjen, H. C. van der Mei, H. J. Busscher, W. Norde, Langmuir 20, 10949 (2004).
5. R. G. Nuzzo, D. L. Allara, J. Am. Chem. Soc. 105, 4481 (1983).
6. M. D. Porter, T. B. Bright, D. L. Allara, C. E. D. Chidsey, J. Am. Chem.Soc. 109, 3559 (1987).
7. E. B. Ttoughton, C. D. Bain, G. M. Whitesides, R. G. Nuzzo, D. L. Allara, M. D. Porter, Langmuir 4, 365 (1988).
8. C. D. Bain, J. Evall, G. M. Whitesides, J. Am. Chem. Soc. 111, 7155 (1989).
9. C. D. Bain, G. M. Whitesides, J. Am. Chem. Soc. 111, 7164 (1989).
10. C. D. Bain, E. B. Troughton, Y. T. Tao, J. Evall, G. M. Whitesides, R. G. Nuzzo, J. Am. Chem. Soc. 111,312 (1989).
11. Y. Li, J. Huang, R. T. McIver. Jr., J. C. Hemminger, J. Am. Chem. Soc. 114, 2428 (1992).
12. Y. Mori, S. Nagaoka, H. Takiuchi, T. Kikuchi, N. Noguchi, H. Tanzawa, Y. Noishiki, Trans. Am. Soc. Artif. Internal. Organs 28, 459 (1982).
13. K. Bergdtrom, E. Osterberg, K. Holmberg, A. S. Hoffman, T. P. Schuman, A. Kozlowski, J. M. Harris, J. Biomater. Sci. Polym. Edn. 6, 123 (1994).

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14. J. M. Harris, Ed. Poly (ethylene glycol) Chemistry, Biotechnical and Biomedical Applications, Plenum Press, New York (1992).
15. J. E. Glass, Ed. Hydrophilic Polymers, Performance with Environmental Acceptance, American Chemical Society, Washington DC (1996).
16. A. Bilic, J. R. Reimers, N. S. Hush, *J. Phys. Chem.* 106, 6740 (2002).
17. D. Y. Wu, B. Ren, Y. X. Jiang, X. Xu, Z. Q. Tian, J. *Phys. Chem.* 106, 9042 (2002).
18. H. Skaff, T. Emrick, *Chem. Commun.* 52 (2003).
19. N. Leventis, I. A. Elder, X. Gao, E. W. Bohannan, C. S. Leventis, A. M. M. Rawashdeh, T. J. Overschmidt, K. R. Gaston, *J. Phys. Chem.* 105, 3663 (2001).
20. K. Uchida, H. Otsuka, M Kaneko, K. Kataoka, Y. Nagasaki, Anal. Chem. 77, 1075 (2005).

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