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Assembly fabrication of linkers on glass surface and their effect on DNA synthesis and hybridization

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Abstract: Linkers were assembled on a glass surface based on the hydrolysis and condensation of 3-glycidoxypropyltrimethoxysilane (GPS). After the assembly of GPS, four approaches were tried to open the ending epoxide group of GPS or to further elongate the linkers. The effect of these approaches on DNA in situ synthesis and hybridization was investigated. For the spacing of the synthesis initiation sites, the wettability of the support and the length of the linking group that attaches the initiation site to the surface have direct influences on the yield of coupling reactions and the subsequent hybridization events. X-ray photoelectron spectroscopy (XPS) and mean contact angles of deionized water of the above slides were measured to assess the linker's characteristics in each procedure. It was proved that the glass slides were successfully modified and became excellent supports for the oligonucleotides synthesis. In addition, it proved best for the in situ oligonucleotides synthesis that a glass slide was in turn treated with ethylenediamine, glutaradehyde, ethanolamine and sodium borohydride solution at ambient temperature after silanized with GPS.

Key words: linker; assembly fabrication; in situ oligonucleotide; synthesis; hybridization

A biochip is a kind of minisized biosensor with many parameters. A group of microanalysis cell and system has been founded by immobilizing thousands of molecular probes on a microminiature solid substrate so that it can make large-scale parallel filtration or measurement of protein and nucleic acid or other bio-components exactly and swiftly[1]. Among them, the gene chip is one of the most important biochips, also named the DNA chip or DNA microarray. With serried oligonuleotide-probes immobilized on the surface and matching the target DNA sequence, DNA microarray technology allows a simultaneous analysis of a large number of nucleic acid hybridization experiments in a rapid and efficient fashion[2]. Two approaches, on-chip synthesis and off-chip synthesis, can be used to prepare microarrays[1]. On-chip synthesis adopted combinatorial chemistry to in situ synthesize for different probes on different chemical coupling sites by a group of orientation templates. The key step is to divide the high density oligonuleotide-probe arrays into template orientation and to in situ synthesize DNA probes on the solid phase. S.P.A. Fodor and coworkers[3] put forward a light directed DNA on-chip synthesis technology, using 10^6 disparate DNA probes which were triumphantly synthesized on a solid substance of 1 cm^2 in terms of combinatorial chemistry. Yet the cost was high and several problems on the efficiency of single-step synthesis remained unsolved. A molecular stamps method developed by Lu Zuhong, et al.[4] adopted commercial DNA synthetic reagents. In recent years, molecular stamps have been systemically studied in depth and a series of research progress has been made[5-7].

In the process of synthesis in situ, oligonuleotide synthesis is closely associated with the density and chain length of active group (linkers) on the carrier. And the nature of linkers also has an impact on the subsequent oligonuleotide hybridisation after synthesis. The surface density of the linkers is expected to be an important parameter for obtaining optimal synthesis and hybridization. A low-surface coverage would presumably yield a low hybridization signal and decrease the hybridization rate. Conversely, a too high-surface density might result in the steric interference between the covalently immobilized probes, impeding their accessibility to the target DNA strands. Furthermore, the chain length of linkers will influence the synthesis as well. If it is too short, it is not feasible for reactant to contact with reaction dot owing to space counteract. And if it is too long, it will be wasteful and linkers will be prone to fold and distort[8,9]. The wettability of a support surface also directly influences the chemical effect of the synthesis, since monomer reactant solution is hydrophilic. On the hydrophobic surface, the hydrophobic sequence will shrink on the surface so that the reactant cannot contact with reaction dot[10]. While on the hydrophilic surface, the reactant can contact

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well with the reaction spots because the surface accords to its physical circumstance.

In this paper manifold methods were adopted to modify a kind of glass slide made in China. The efficiency of oligonuleotide synthesis and subsequent hybridisation have been improved through optimizing the conditions, adjusting the density of linkers and properly increasing the chain length of linkers.

1 Experimental Sections

1.1 Chemicals

Ar (99.995%) and 25% glutaradehyde solution in water were purchased from local stores. 3-glycidoxypropyltrimethoxysilane (GPS) and hexaethylene glycol were purchased from Tokyo Kasei KOGYO Co. Ltd (Japan). The oligonucleotide synthesis reagents and solvents were purchased from PE Biosystems except for the oxidation reagents prepared by ourselves. The used fluoremonomer with a hexachloro-6-carboxyl-fluorescein (HEX) was purchased from Shanghai Biological Produce Corporation. Other chemical reagents were analytically pure and purchased from local stores.

1.2 Method

1.2.1 Cleaning of slides and measurement of contact angles

The glass substrates used in this study were standard "precleaned" soda lime microscope slides of Fanchuan brand (25.4 mm × 76.2 mm × 1 mm, Cat. NO.7105, Cat.NO.7101, China). Chemical cleaning methods of slides used are shown in Tab.1[11]. Its cleaning effect can be ascertained by measurement of contact angles on the slides surface. Equilibrium contact angle measurements were performed using a Rame-Hart-100 contact angle instrument. Measurements were made on sessile drops (1 μL droplets) by measuring thetangent to the drop at its intersection with the slide surface: Values obtained were the mean of the tangents taken at both sides of each droplet.

Tab.1 Cleaning methods and contact angles

Method	Steps	Contact angle (± 5°)/(°)
*	Slides untreated	37
1	30 min in 1 : 1 (volume ratio) MeOH : HCl, rinse in H_2O, dry under Ar	15
2	Method 1 + additional incubation (30 min) in concentrate H_2SO_4, rinse in H_2O, dry under Ar	18
3	Method 2 + additional incubation (30 min) in 100 ℃ H_2O, dry under Ar	12

1.2.2 The modification of the glass slides

Method 1 The chemistry principle used is shown in Fig.1. The detailed steps followed: ① The cleaned slides were suspended in a solution of 5% of GPS in water, keeping the pH between 5.5 and 5.8. After reaction for 30 min at 90 ℃ the slide was rinsed using ethanol. ② A dilute solution of HCl in water was used to cleave the epoxide residue to yield a primary hydroxyl group, then the slide was rinsed in deionized water and air dried for oligonucleotides synthesis[12].

Method 2 The chemistry principle used is shown in Fig.1 and Fig.2. The detailed steps followed: ① The cleaned slides were suspended in a solution of 5% of GPS in water at pH between 5.5 and 5.8 for 30 min at 90 ℃ and then rinsed using ethanol. ② The slides were heated with stirring in hexaethylene gycol, containing a catalytic amount of concentrate sulfuric acid, overnight at 80 ℃ to yield the alkyl hydroxyl modified supports. Then the slides were rinsed in ethanol. ③ The slides were treated with a dilute solu-

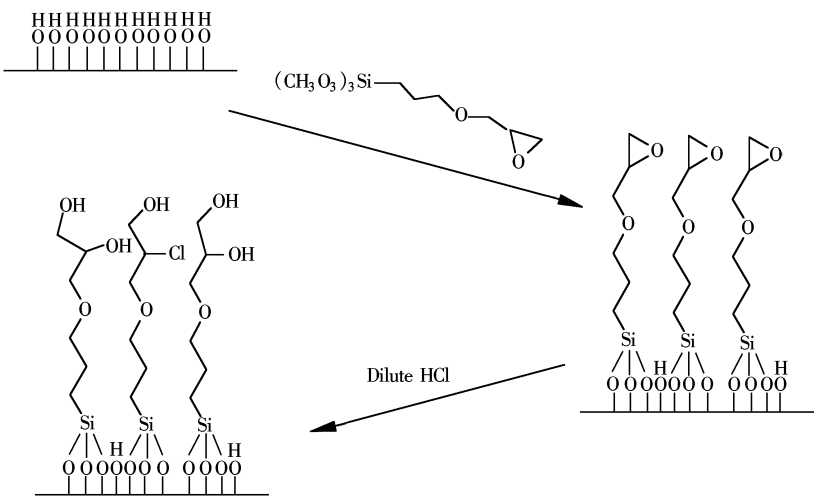

Fig.1 Chemical process of silanization by 3-glycidoxypropyltrimethoxysilane and hydrolyzation by hydrochloric acid

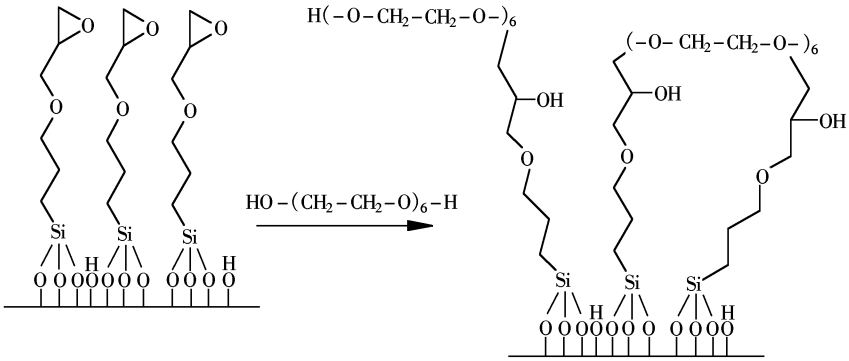

Fig.2 Chemical process of silanization by 3-glycidoxypropyltrimethoxysilane and reaction with hexaethylene glycol

tion of HCl in water to cleave the epoxide residue to yield a primary hydroxyl group, then rinsed in deionized water and dried air.

Method 3 The chemistry principle used is shown in Fig.3. The detailed steps followed: ① The cleaned slides were suspended in a solution of 5% of GPS in water under the pH ranging from 5.5 to 5.8 at 90 ℃ for 30 min, then rinsed using ethanol. ② The slides were treated with 10% ethylenediamine for 2 h, 5% glutaradehyde in phosphate buffered saline (PBS) (0.1 mol/L, pH = 7.4) for 2 h, 10% ethanolamine for 2 h, and $NaBH_4$ solution ($NaBH_4$ 0.07 g, 10 × PBS 2 mL, ethanol 6 mL, distilled water 17 mL) for 15 min at ambient temperature, respectively[13].

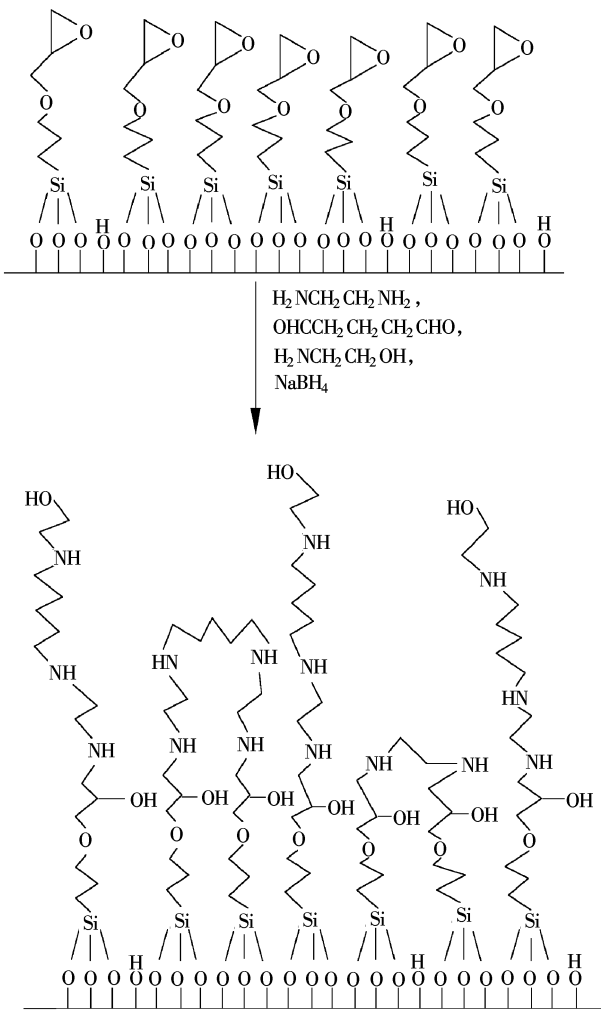

Fig.3 Chemical process of silanization by 3-glycidoxypropyltrimethoxysilane and the possible surface modification reaction in method 3

Method 4 The chemistry principle used is similar to that in Fig.3 for method 3, except for substituting hexaethylene gycol for ethylenediamine.

1.2.3 Olignuleotides synthesis and detection

The manual coupling reaction was conducted in a glove-box (Mecaplex, Switzerland); the concentrations (by volume) of H_2O and O_2 in the glove-box were below 0.000 2% and 0.001 5%, respectively. A reagent containing fluorescence monomer and tetrazole was dripped on the slide with a dropper. Then the glass slide was shaken in a mixed solution of ethanol and ethanol amine (volume ratio is 1 : 1) in a sealed box at 75 ℃ for 2 h for deprotection. Then it was washed with distilled water and dried by cold blowing before detection or hybridization. An image of the fluorescence signal was obtained with Scan-Array Lite Microarray Analysis System (Packard Biochip Technologies), and the fluorescence intensity was analyzed with the software in the scanner[6]. The automatic oligonucleotide synthesis was carried out on the Model 391 DNA Synthesizer (Applied Biosystems). A predesigned groove was chiseled out on a flat polydimethylsiloxane (PDMS) film; synthetic region on the glass slide was jointed with the groove. A teflon plate with entry and exit plastic tubes was covered on the carved PDMS film. The sandwich was fixed with iron clamps, and formed a closed fluidic system. Oligonucleotides (5'-AGG AGG CTA AGT CTC CTA GG-3') synthesis was carried out on the glass slide.

2 Results and Discussions

2.1 Cleaning of slides

The uniform deposition of a silane monolayer upon

glass has been shown to require a small amount of water along with hydroxyl groups in an isolated or geminal configuration on the substrate surface. In order to expose these groups, inorganic or organic compounds at the surface must be removed. J.J.Cras and coworkers have made detailed research[11]. On the basis of their work, the "precleaned" microscope slides were chemically cleaned by manifold methods. The result of treatment is shown in Tab.1.

Goniometric analysis provides a relatively quick and simple means of assessing the cleanliness of a glass surface. As seen in Tab.1, all the cleaning methods produced a very hydrophilic surface, but the contact angles were slightly bigger than those reported in Ref. [11], possibly because the treated slides had been stored too long before the contact angle measurement. Tab.1 also demonstrates that the treatment of method 1 was better than that of method 2. The reason probably was that sulfuric acid would react with metallic contaminants and cover the hydroxide group, increasing the contact angle accordingly. While the HCl solution in water would not produce such a negative effect owing to the strong volatility of HCl. However, according to the observation by J.J.Cras[11], method 2 would be superior to method 1 because the slide was cleaned drastically after the sulfuric acid treatment. Therefore, we adopted method 3 in which the wash with a solution of 1 : 1 (volume ratio) methanol to HCl was followed by an acidic bath in concentrated H_2SO_4 to remove surface contaminants followed by an additional incubation in water at 100 ℃. Finally the treated slide was rinsed sufficiently. The results indicated that method 3 can clean the slide most efficiently.

2.2 Characteristics of different linkers

To examine the assembling of linkers on the slide surface, X-ray photoelectron spectroscope (XPS) was used to analyze linkers selectively assembled by different methods. The results are shown in Tab.2[14].

Tab.2 Atomic contents from XPS analysis for linkers by different assembly methods

Atom	Atomic contents/%			
	Untreated	Method 1	Method 3	Method 4
C	26.52	31.84	29.07	31.26
O	51.98	47.92	49.06	47.17
Si	20.65	19.32	18.80	19.68
N	0.84	0.92	3.07	1.88

The nitrogen content on the surface of the slides modified by method 1 was very close to that of the untreated one on which amidogen had not been linked by method 1. However, owing to the introduction of a nitrogenous compound, ethanolamine, in method 4, the nitrogen content increased to 1.88%. Furthermore the nitrogen content reached 3.07% by method 3 because of the introduction of ethylenediamine rich in nitrogen. The above results are identical with the chemical process for the assembling of the linkers, and synchronously indicate the successful assembling of linkers.

To examine the hydrophility of linkers, contact angles were measured and the results are shown in Tab. 3. According to Ref. [11], contact angles increased after silanization.

Tab.3 Hydrophility of linkers assembled by distinct methods

Treatment	Contact angle (± 5°)/(°)
Method 1	15
Method 2	19
Method 3	26
Method 4	33

2.3 Impact of linkers' surface chemistry on olignuleotides synthesis and hybridization

2.3.1 Impact of density of linkers

Distinct linkers were coupled by direct oligonucleotides synthsis in the following three processes: ① Slides were directly coupled by monomer with fluorescence group; ② Slides were coupled with fluorescence group without being catalyzed by tetrazole; ③ The in situ synthesized 20 mer oligonucleotides were hybridized to the complementary target sequences. In the first and second processes, the fluorescence intensity of different slides arranged in a descending order: non-assembled linkers, assembled by method 1, assembled by method 2, assembled by method 3 and assembled by method 4. While in the third process, in which the 20 mer oligonucleotides were in situ synthesized and hybridized with complementary target sequences, fluorescence intensity of distinct slides arranged in a descending order: assembled by method 3, assembled by method 2, assembled by method 1, assembled by method 4 and non-assembled linkers.

The above results can be interpreted by the steric interferences. The surface density of the reactive groups of linkers has an impact on the reactive velocity. However, in the process of directly coupling the monomer with a fluorescence label or directly coupling the monomer with a fluorescence tag without being catalyzed by tetrazole, the higher the density, the more the coupled fluorescence monomers and the greater the fluorescence intensity since only a single coupling step is required for 30 min. As evidence, the

density of the active groups after the assembling of the linkers arranged in a descending order: non-assembled linkers, assembled by method 1, assembled by method 2, assembled by method 3 ≈ assembled by method 4. However, as to the fluorescence intensity of the hybridization of 20 mer oligonucleotides to the complementary target sequence, it is another case as the fluorescence intensity lies entirely on the number of the target molecules hybridized with 20 mer oligonucleotides. On the one hand, the high density of the linkers might impede the nucleotide to access the active groups on the slide surface, decreasing the coupling efficiency. On the other hand, a high surface density might result in steric interference between the covalently immobilized probes, impeding those available to the target DNA strands. As displayed in Fig.3, not only the linker lengths of method 3 are greater than those in Fig.1 and Fig.2 for methods 1 and 2, respectively, but also the density of hydroxide groups for method 3 is lower than for those in Fig.1 and Fig.2, owing to an ethylenediamine or glutaradehyde molecule which may be coupled by two $-NH_2$ groups as shown in Fig.3. The relatively low density of hydeoxide groups would increase the coupling efficiency of the in situ synthesis or hybridization. Therefore, the greatest fluorescence intensity of the hybridization of the 20 mer oligonucleotides to their complementary target sequences in Fig.4 should be mainly contributed by the small steric interferences of the linkers and surface probes.

2.3.2 Impact of linkers length

The fluorescence intensity of hybridization of 20 mer oligonucleotides to complementary target sequence in Fig.4 can also be well explained with the influence of the length of linkers. The length of linkers is increased in turn when assembled by method 1, method 2 and method 3, respectively. With the increase of the length of linkers, the fluorescence intensity of hybridization of 20 mer oligonucleotides to the complementary target sequence increased synchronously. It can be interpreted with collision theory. The active hydroxide group of long linkers has more opportunity to contact and hybridize with the target sequence far away from the surface and more actively, so that the fluorescence intensity is stronger. But why is the fluorescence intensity of method 4 weaker than that of method 1 regardless of the longer linkers of method 4 than that of method 1? It is presumably suggested that too many chemical steps in the process of method 4 make the surface density of hydroxide groups much lower than that of the method 1. A too low surface hydroxide group coverage would significantly decrease the amount of the in situ synthesized 20 mer oligonucleotides and, therefore, the fluorescence intensity would be low even if the hybridization efficiency were high. As to the untreated glass slides, the active hydroxides are too close to the surface. The coupling efficiency will be so low that only a small amount of the in situ synthesized oligonucleotides can give out a fluorescence signal.

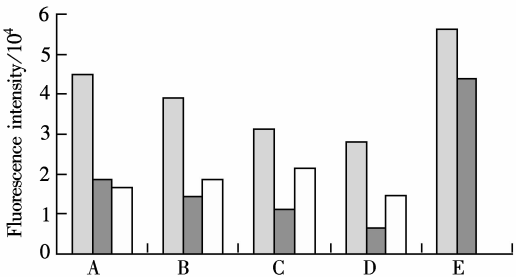

Fig.4 Fluorescence intensity of different linkers by direct oligonucleotides synthesis

In Fig.4, the three processes were that the slides were directly coupled monomer with fluorescence group (30 min), fluorescence group without catalyzed by tetrazole (30 min) and hybridization of 20 mer oligonucleotides to complementary target sequence, respectively. A, B, C and D represent four different methods, respectively (see Methods). E represents control (namely, the slide was not assembled linker).

2.3.3 Accessibility of solvent

The fluorescence intensity of hybridization of 20 mer oligonucleotides to the complementary target sequence in Fig.4 can also be analyzed by the hydrophilicity of linkers. The wettability of a substrate surface will directly give an impact on the coupling and the hybridization. Since monomer reactant solution is hydrophilic, the hydrophobic sequence will shrink on the hydrophobic surface so that the reactant cannot contact the reaction sites. While on the hydrophilic surface, reactants can contact well with reaction sites because the surface accords to its physical circumstance. Tab.3 indicates the linker of method 3 is more hydrophilic than that of method 4 and, accordingly, the fluorescence intensity of hybridization of 20 mer oligonucleotides to the complementary target sequence is stronger than that of method 4. Therefore, it is believed that the difference in surface hydrophilicity also contributes to the strong fluorescence intensity of hybridization of method 3.

3 Conclusion

The above experiments proved that the glass slides

were successfully modified and became excellent supports for oligonucleotides synthesis. In addition, under our experiments the most feasible method for the in situ synthesis of oligonucleotides was treating glass slides with ethylenediamine, glutaradehyde, ethanolamine and sodium borohydride solution in sequence at ambient temperature after the silanization with GPS.

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玻片表面手臂分子的组装及对寡核苷酸原位合成与杂交的影响

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摘　要　以3-缩水甘油-环氧丙基-三甲氧基硅烷对玻璃基片表面进行硅烷化后,用下列4种方法组装手臂分子:① 盐酸直接处理;② 先用聚六乙二醇,然后用盐酸处理;③ 用乙二胺、戊二醛、乙醇胺和硼氢化钠分别处理;④ 用聚六乙二醇、乙二胺、戊二醛、乙醇胺和硼氢化钠分别处理.用XPS (X-ray photoelectron spectroscopy) 和测定接触角的方法对上述组装进行了表征,并用直接偶联荧光单体及合成20 mer寡核苷酸与带荧光的互补探针杂交的方法对上述手臂分子的合成效率及杂交效率进行了考察.实验表明方法③组装的手臂分子得到的结果优于其他3种方法,证明了手臂分子的空间效应、亲水性等性质对寡核苷酸合成和杂交存在影响.

关键词　手臂分子; 组装; 寡核苷酸; 合成; 杂交

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