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COUPLING OF LIGANDS TO PRIMARY HYDROXYL-CONTAINING SILICA FOR HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

OPTIMIZATION OF CONDITIONS

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SUMMARY

Silicas of different particle and pore sizes were derivatized with three different silanes. The functionalized silica contained either epoxide, methacrylate or amino groups. These groups were further modified to yield primary hydroxyl functions. Activation of the resultant primary hydroxyl groups for the purpose of chemically coupling proteins was studied with a variety of reagents and optimized for *p*-nitrophenyl chloroformate. The effect of pH on the efficiency of coupling proteins (BSA and trypsin) to *p*-nitrophenyl carbonate-silica was studied in detail. Slightly acidic conditions (pH 6) gave the highest yields. In a dynamic recycling process, bovine pancreatic trypsin inhibitor was immobilized to activated primary hydroxyl-silica packed into a stainless-steel column. The high-performance affinity chromatography purification of trypsin on this column is demonstrated.

INTRODUCTION

Since the development of reversed-phase chromatography, chemically modified silica has been used as a column material for the chromatography of biologically active compounds. However, irreversible adsorption due to hydrophobic interactions and denaturation, caused by silanol groups on the silica surface, have limited the use of silica for this purpose in many instances[1].

Derivatization of the silanol groups with 3-glycidoxypropyltrimethoxysilane has provided, after hydrolysis of the epoxide functions, a hydrophilic diol-silica. The latter was shown to be suitable for the chromatography of biological macromolecules[2]. Diol-silica has also been used as a carrier for high-performance affinity chromatography (HPAC)[3]. However, the coupling capacity of the ligand to the activated diol-silica is not high, owing to the possible formation of a five-membered stable cyclic carbonate. Subsequent oxidation of the diol-silica yields aldehyde-silica, which has also been employed for HPAC[4]. Although the coupling of ligands to this support proceeds with high yields, the resultant Schiff base formed between ligand and carrier is not stable if not reduced. In many instances reduction causes a loss of biological activity. Reduction of the aldehyde-silica leads to primary hydroxyl-silica, which we have recently shown to be an excellent support for HPAC[5].

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In this paper, we describe different methods for the chemical modification of silica, which generate primary hydroxyl groups. Their activation with various reagents (chloroformates, 1,1,1-trichloro-2-monochloroethylsuccinimide carbonate, carbodiimidazole and sulphonyl chlorides) is also presented. The conditions for activation and coupling of the ligands were optimized.

EXPERIMENTAL

Materials

Porous silica (LiChrosorb Si 60, 10 μm, 60 Å; LiChrosorb Si 100, 10 μm, 100 Å; Kieselgel 60, 40–63 μm, 60 Å) and *p*-toluenesulphonyl chloride were obtained from Merck (Darmstadt, F.R.G.); Partisil 5, 5 μm, 60 Å, was obtained from Whatman (Maidstone, U.K.). 3-Glycidoxypropyltrimethoxysilane, 3-methacryloxypropyltrimethoxysilane, 3-aminopropyltriethoxysilane, *p*-nitrophenyl chloroformate and 1,1′-carbodiimidazole were purchased from Aldrich (Milwaukee, WI, U.S.A.). 1,1,1-Trichloro-2-monochloroethylsuccinimide carbonate was kindly provided by Propeptide (Paris, France). N-Hydroxysuccinimide chloroformate was synthesized according to Gross and Bilk[6]. 4-Dimethylaminopyridine, bovine serum albumin (BSA), bovine pancreate trypsin inhibitor (BPTI) and trypsin were obtained from Sigma (St. Louis, MO, U.S.A.).

Modification of the silica surface with silanes

Silanization of silica (Partisil 5, LiChrosorb Si 60 and Kieselgel 60) with 3-glycidoxypropyltrimethoxysilane (Fig. 1a) was carried out under anhydrous conditions according to Larsson *et al.*[4]. LiChrosorb Si 60 was derivatized by the same procedure with 3-methacryloxypropyltrimethosysilane (Fig. 1b). For the preparation of 3-aminopropyl-silica (Fig. 1c) we used LiChrosorb Si 100 as a starting material and modified the reaction procedure as follows. To a suspension of 5 g of LiChrosorb

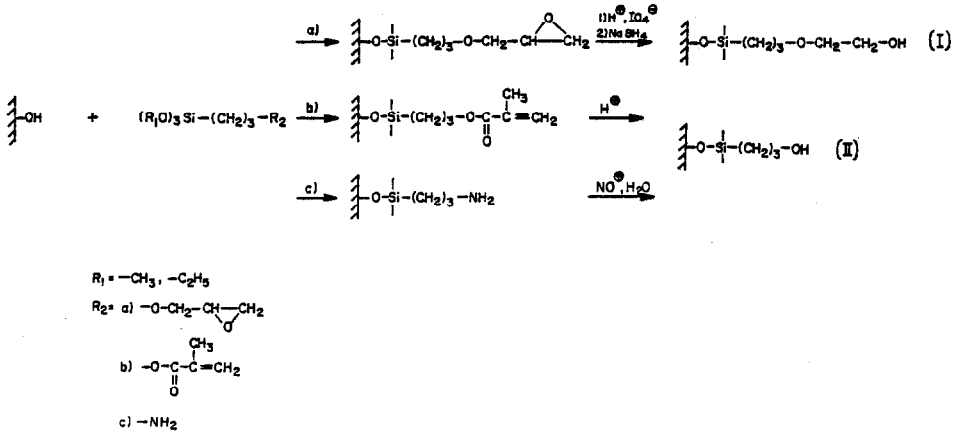


Fig. 1. Chemical modification of silica with (a) 3-glycidoxypropyltrimethoxysilane, (b) 3-methacryloxypropyltrimethoxysilane and (c) 3-aminopropyltriethoxysilane, leading to primary hydroxyethyloxypropyl-silica (I) and hydroxypropyl-silica (II).

Si 100 in 150 ml of dried (over sodium) toluene, 25 mmol of silane and an equimolar amount of triethylamine were added. The reaction mixture was stirred and refluxed for 24 h. After washing the silica with toluene, acetone and diethyl ether, the resin was dried under vacuum. The degree of silanization on the silica surface was determined by titration[4] for the epoxide-silica and by elemental analysis for methacrylate- and amino-silicas.

Preparation of primary hydroxyl-silica

Hydroxyethyloxypropyl-silica (Fig. 1, I). Hydrolysis of 3-glycidoxypropyl-silica with acid afforded diol-silica, which was then oxidized to yield aldehyde-silica[4]; subsequent reduction led to the production of primary hydroxyl groups[5].

Hydroxypropyl-silica (Fig. 1, II). (i) 3-Methacryloxypropyl silica (5 g) was suspended in 100 ml of 1 *M* hydrochloric acid and stirred at 60°C for several hours. (ii) 3-Aminopropyl-silica (3 g) was suspended in 300 ml of 6 *M* hydrochloric acid, stirred and cooled in an ice-bath, and a solution of 1.5 *M* sodium nitrite (30 ml) was added dropwise. The suspension was then heated until evolution of nitrous gases ceased. The reaction was followed with ninhydrin.

Activation of primary hydroxyl-silica (Fig. 2)

Chloroformates (p-nitrophenyl- and N-hydroxysuccinimide chloroformates[7]). To a suspension of 150 mg of primary hydroxyl-silica in 5 ml of dried (over potassium carbonate) acetone, the desired amount of chloroformate was added (the amount used for time- and base-dependence studies was 1.5 mmol). The mixture was cooled and stirred in an ice-bath and a 0.5 mmol excess of base (if not stated otherwise, 4-dimethylaminopyridine was used) dissolved in 5 ml of dry acetone was added dropwise. The reaction was continued for 30 min, unless indicated otherwise. Subsequently, the silica was washed successively on a sintered funnel with acetone, 5% acetic acid in dioxane, acetone and diethyl ether. The product was dried under vacuum.

1,1,1-Trichloro-2-monochloroethylsuccinimide carbonate. Primary hydroxyl-silica (150 mg) and 1,1,1-trichloro-2-monochloroethylsuccinimide carbonate (1.5 mmol) were suspended in 5 ml of dry acetone. While the mixture was cooled and stirred in an ice-bath, 2 mmol of 4-dimethylaminopyridine dissolved in 5 ml of dry acetone was added dropwise. Washing of the silica was performed as described above.

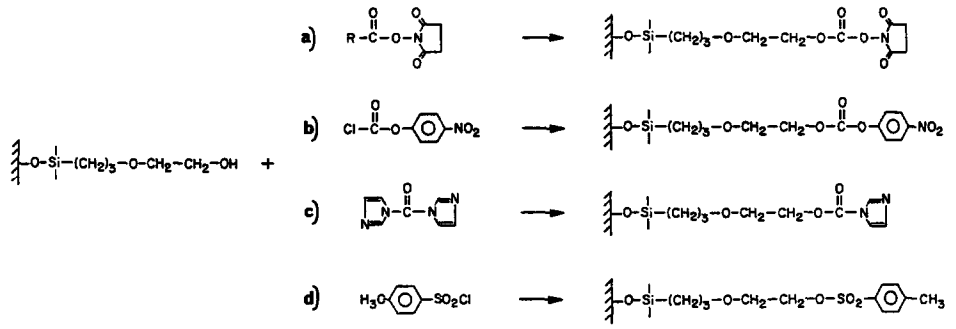


Fig. 2. Activation of primary hydroxyl-containing silica with (a) N-hydroxysuccinimide chloroformate (R = Cl) and 1,1,1-trichloro-2-monochloroethylsuccinimide carbonate [R = $Cl_3CCH(Cl)O$–); (b) *p*-nitrophenyl chloroformate; (c) 1,1'-carbodiimidazole; and (d) *p*-toluenesulphonyl chloride.

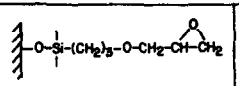
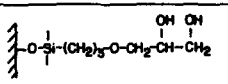
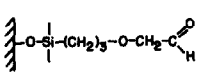
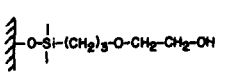
	Epoxide silica	Diol silica	Aldehyde silica	Primary hydroxyl silica
Structure				
Activation of functional groups	———	a) Tresylchloride b) 1,1'-carbodiimidazole	———	a) Tresylchloride b) chloroformates,1,1'carbodiimidazole
pH of coupling	alkaline, > 8.0	physiological, 7.4	acidic, < 6.0	physiological,7.4
Time of coupling	~7 days	1-2 days	2 days	0.5-2 days
Capacity of coupled ligand	very low	low	high	high
Stability of covalent attachment of ligand	stable	stable	not stable reduction with $NaCNBH_3$	stable
Introduction of charged groups after removal of excess of active groups	yes	a) yes	amine yes reduction no	a) yes b) no

Fig. 3. Comparison of the properties of epoxide-, diol-, aldehyde- and primary hydroxyl-silica as carriers for the immobilization of proteins. Tresyl chloride = $CF_3CH_2SO_2Cl$.

1,1'-Carbodiimidazole. Activation with 1,1'-carbodiimidazole[3] was carried out by preparing a suspension of 150 mg of silica and 1.5 mmol of 1,1'-carbodiimidazole in 5 ml of dry dioxane. The reaction mixture was stirred for 30 min at room temperature. Washing of the silica was performed with dioxane, acetone and diethyl ether.

Tosyl (or tresyl) chloride[8]. For the preparation of tosylated (or tresylated) silica, 150 mg of silica and 1.5 mmol of *p*-toluenesulphonyl (or tresyl) chloride were suspended in 5 ml of dry acetone. After the addition of 5 mmol of pyridine, the reaction was continued for 1 h at room temperature with continuous stirring. The silica was washed according to the procedure described above.

Determination of active groups on the silica

The amount of active groups on the silica, activated with chloroformates and 1,1,1-trichloro-2-monochloroethylsuccinimide carbonate, was determined spectrophotometrically after basic hydrolysis, as described previously[7]. The amount of imidazolyl and tosyl groups was determined by elemental analysis.

Coupling of ligands to the activated carrier

Activated silica (100 mg) was suspended in 5 ml of 0.1 *M* phosphate buffer (pH 7) and either 5 mg of BSA or 10 mg of trypsin were added. The suspension was then gently shaken or stirred at 4°C for 2 days. The resultant silica was washed with 0.1 *M* phosphate buffer. The amount of coupled protein was determined by amino acid analysis.

Chromatographic procedure

The chromatographic set-up used was as described previously[5].

Coupling of ligands to pre-packed activated columns

A stainless-steel column (40 × 4.6 mm I.D.) was packed with about 0.5 g of N-hydroxysuccinimide chloroformate-activated silica. A solution of 29 mg of BPTI in 10 ml of water was recycled for 7 h through the column. The absorbance of the ligand solution was measured during the coupling process to determine the amount of bound BPTI. For this experiment, N-hydroxysuccinimide-activated silica was used instead of *p*-nitrophenyl carbonate, as the amount of protein coupled could be followed directly by measuring the UV absorption. In order to remove the excess of active groups, 30 ml of 0.2 *M* ammonia solution were pumped through the column for 30 min.

RESULTS AND DISCUSSION

In preliminary studies[5], we used a four-step synthesis to introduce primary hydroxyl groups into silica by employing a commonly used reagent (3-glycidoxypropyltrimethoxysilane) as a surface modifier[2]. The advantage of this silane is that each intermediate product (epoxide-, diol- and aldehyde-silica) obtained during the synthesis of the corresponding primary hydroxyl-silica can be used for the subsequent immobilization of ligands. The coupling behaviour of these intermediate derivatives was compared with two model proteins, BSA and trypsin. As summarized in Fig. 3,

the epoxide showed only slight reactivity towards the protein, whereas the aldehyde bound a significant amount of ligand. However, coupling of amino-containing ligands to aldehydes yields a labile Schiff base which cause leakage of the ligand. In contrast, immobilization of ligands on primary hydroxyl groups was performed in high yields under mild conditions. We therefore decided to concentrate our studies on additional efficient methods for the chemical incorporation of primary hydroxyl groups into the silica surface.

In this study, we used 3-methacryloxypropyltrimethoxysilane and 3-aminopropyltriethoxysilane as surface modifiers; hydrolysis of the methacrylate or diazotisation of the amino-silica both result in primary hydroxyl silica (see Fig. 1).

The amount of hydroxyl groups activated with *p*-nitrophenyl chloroformate on the different types of modified silicas is given in Table I. Although the ligand density of primary hydroxyl groups varied considerably for these silicas, the amount of activated groups with *p*-nitrophenyl chloroformate (220–280 μmol/g) was similar for all the silicas. Lower activation levels were obtained only with silica derivatized with 3-methacryloxypropyltrimethoxysilane, probably because hydrolysis of the methacrylate silica was not complete. Interestingly, the aminopropyl-silica can also be activated with chloroformates in high yields (about 440 μmol/g), and high coupling yields of proteins can be obtained, *e.g.*, 57.6 mg of BSA and 82.5 mg of trypsin per gram of silica. Nevertheless, we prefer the hydroxyl-silica, as no charged groups are left after the coupling of the proteins and cross-linking is minimized.

The degree of activation and the coupling capacities generated by various activating agents are given in Table II. Although 1,1′-carbodiimidazole appeared to give high levels of activation, the coupling yield of trypsin was similar to the *p*-nitrophenyl chloroformate-activated silica, indicating that the *p*-nitrophenyl carbonate has a higher activity towards nucleophiles and is less hydrolysable. In order to determine the optimal coupling conditions (*i.e.*, low rate of hydrolysis but high reactivity towards the ligand), we coupled trypsin and BSA to *p*-nitrophenyl chloroformate-activated resins under various pH conditions. As can be seen in Fig. 4, the maximum amount of coupled trypsin is obtained between pH 6 and 7; the amount

TABLE I

CHARACTERIZATION OF SILICAS MODIFIED WITH DIFFERENT SILANES

Silica	*Particle size (μm)*	*Pore size (Å)*	*Silane surface modifier**	*Ligand density (μmol/g)*	*Activated groups*** *(μg/g)*
Partisil 5	5	60	GPS	360	255
LiChrosorb Si 60	10	60	GPS	380	281
LiChrosorb Si 60	10	60	MPS	710	123
LiChrosorb Si 100	10	100	APS	900	437*** 223§
Kieselgel 60	40–63	60	GPS	450	261

* Silanes used as surface modifiers: GPS, 3-glycidoxypropyltrimethoxysilane; MPS, 3-methacryloxypropyltrimethoxysilane; APS, 3-aminopropyltriethoxysilane.

** The amount of activated groups was determined by activation with *p*-nitrophenyl chloroformate.

*** Activation of amino functions on the silica surface.

§ Activation of hydroxyl functions after diazotization of the amino-silica.

TABLE II

ACTIVATION OF HYDROXYETHYLOXYPROPYL-SILICA OF LICHROSORB Si 60 WITH DIFFERENT REAGENTS: DETERMINATION OF ACTIVE GROUPS AND COUPLED TRYPSIN

Activating reagent	*Activated groups (μmol/g)*	*Coupled trypsin (mg/g)*
p-Nitrophenyl chloroformate	282	50.5
N-Hydroxysuccinimide chloroformate	320	47.1
1,1,1-Trichloro-2-monochloroethyl-succinimide carbonate	129	33.8
1,1'-Carbodiimidazole	480	51.5
p-Toluenesulphonyl chloride	363	14.6

of coupled BSA is relatively constant between pH 4 and 6, with a maximum at pH 6. A significant reduction in coupling capacities is observed at higher pH. These results are in agreement with those recently reported by Crowley *et al.*[9], who showed that the most efficient coupling of proteins on carbodiimidazole-activated resins takes place at pH 4–5. Both results clearly indicate that it is preferable not to use basic conditions for coupling ligands to activated carbonates[10,11].

In order to increase the yields of both activated groups and coupled ligands further, we carried out a series of experiments with *p*-nitrophenyl chloroformate. In these studies we varied (a) the time of activation, (b) the concentration of chloroformate and (c) the type of base that catalyses the reaction. As shown in Fig. 5, the activation of the hydroxyl function is strongly time dependent. The amount of activated groups decreases almost exponentially after 1 h, indicating that considerable hydrolysis or displacement (see Fig. 6) of the newly formed *p*-nitrophenyl carbonate takes place. Fig. 7 demonstrates that the efficiency of activation is dependent on the concentration of *p*-nitrophenyl chloroformate added. Activation was performed by increasing the amount of chloroformate stepwise. Using a 30-fold excess of activation reagent (2 mmol of chloroformate for 150 mg of Kieselgel 60), the amount of acti-

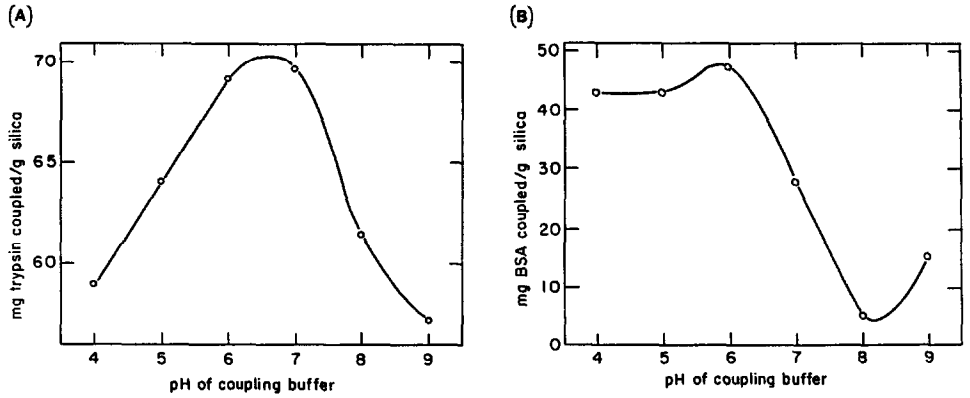


Fig. 4. pH dependence of coupling (A) trypsin and (B) BSA to *p*-nitrophenyl chloroformate-activated primary hydroxyl-silica with hydroxyethyloxypropyl-silica of LiChrosorb Si 60 and 0.1 *M* phosphate buffer.

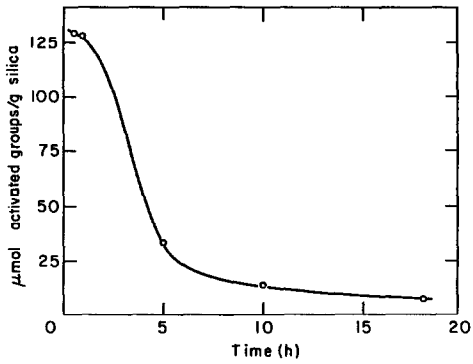


Fig. 5. Time dependence of the activation process with *p*-nitrophenyl chloroformate- and hydroxyethyl-oxypropyl-silica of LiChrosorb Si 60.

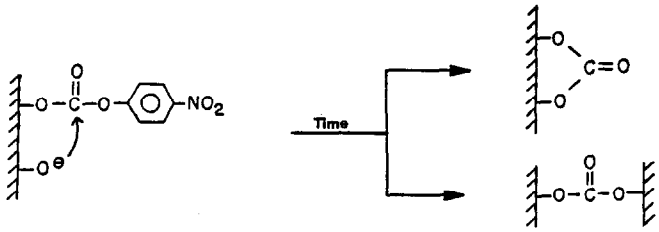


Fig. 6. Displacement of *p*-nitrophenyl carbonate by nucleophilic attack of hydroxyl groups on the silica, forming carbonate derivatives.

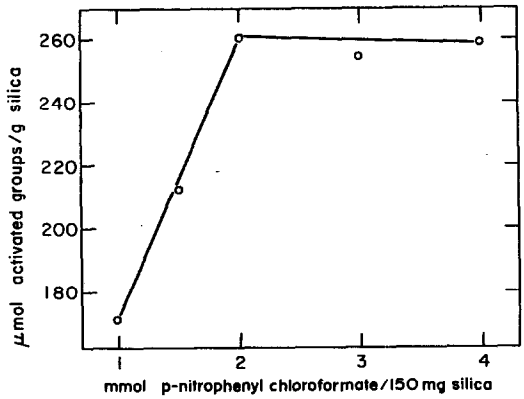


Fig. 7. Amount of activated groups as a function of added *p*-nitrophenyl chloroformate with hydroxy-ethyloxypropyl-silica of Kieselgel 60.

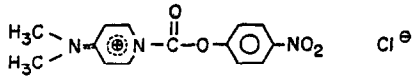


Fig. 8. Intermediate complex formed with 4-dimethylaminopyridine and *p*-nitrophenyl chloroformate.

TABLE III

INFLUENCE OF BASE OF THE DEGREE OF ACTIVATION USING *p*-NITROPHENYLCHLOROFORMATE AND HYDROXYETHYLOXYPROPYL-SILICA OF KIESELGEL-60

Base	*Activated groups (μmol/g)*
Triethylamine	130.6
Pyridine	217.6
4-Dimethylaminopyridine	282.3

vated groups reached a plateau. In a third series of experiments, we studied the effect of different bases on the activation reaction with *p*-nitrophenyl chloroformate (Table III). The most suitable reagent was 4-dimethylaminopyridine, which forms an intermediate complex with the chloroformate[12], thus increasing the reactivity of the chloroformate toward the hydroxyl function (Fig. 8).

A clear advantage of the use of HPAC is the time factor. High pressures and high flow-rates allow the purification of biological material in a short time. Another advantage is the possible immobilization of ligands on activated resins in a dynamic process. Ligands can be immobilized on the activated silica before packing into the stainless-steel column or after packing the column with the activated silica.

In a typical experiment, we filled a column with activated silica and pumped through the column, in a dynamic recycling process, a solution containing BPTI as a ligand. The amount of BPTI coupled was followed spectrophotometrically and did not change significantly after 7 h. It was determined as 16 mg per gram of silica. Trypsin was then adsorbed on and eluted from the resultant affinity column. This finding demonstrates that ready-made activated columns for coupling different proteins and ligands are stable and can be prepared for laboratories that do not have a column-filling capability.

It is expected that if high-capacity and efficient silica columns were commercially available, more laboratories would shift from affinity chromatography to HPAC, as the columns are smaller, the handling times are shorter and the cost is lower.

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