Optically Controlled Ligand Delivery. II. Copolymers Containing α-Methylphenacyl Bonds

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SYNOPSIS

N-(2-hydroxypropyl)methacrylamide copolymers containing side chains terminated in a model ligand, *N-tert*-butyloxycarbonylglycine (Boc-Gly), bound via photocleavable α-methylphenacyl bonds were synthesized to test the possibility of developing an optically controlled ligand delivery system suitable for sensors. One of the copolymers was further covalently attached to 3-aminopropyl triethoxy silane (APS) coated porous silica beads which were modified with an excess of α,ω-diaminopoly(ethylene oxide), molecular weights 1000 or 5000. The photochemical release of ligand induced by exposure to light in solution and at the solid–liquid interface was studied. The influence of solvent and the length of poly(ethylene oxide) (PEO) spacers on the rate of photocleavage were determined. The hydrolytic stability of the α-methylphenacyl bond in both solution and at the solid–liquid interface were also investigated.

INTRODUCTION

The specificity of antibody (Ab)–antigen (Ag) interactions and competitive binding reactions using labeled Ag or Ab have permitted the development of immunoassays with very high sensitivity and specificity. Although modern competitive binding immunoassay kits are very simple to use, they are totally unsuitable for continuous and/or remote measurements.

There exists a possibility to use the competitive binding immunoassay principle in fiber optics and thin film wave guide sensors.[1] Such sensors will be based on remotely controlled ligand delivery and total internal reflection fluorescence (TIRF) sensing. The development of such sensors requires a number of basic and applied science studies[2] including polymer synthesis, remote ligand delivery,[3] antibody binding, Ag–Ab binding constant regulation,[4] and biocompatibility.

One of the key parts of the overall sensor problem is the optically controlled release of ligands. To investigate this question, we have studied *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing side chains terminated in ligands bound via photocleavable bonds. Copolymers of HPMA were chosen because their structures permitted introduction of different reactive units into one polymeric chain[5] and, consequently, offer the possibilities to attach ligand-containing copolymers to functionalized surfaces.

In the first paper of this series HPMA copolymers with ligands (Boc-Gly, fluorescein, and tetramethylrhodamine) bound via the 2-nitrobenzyl bond were synthesized and the photocleavage studied.[3] From these experiments, it may be concluded that Boc-Gly is stable during irradiation and photocleavage, whereas fluorescein and tetramethylrhodamine undergo structural changes when in solution. However, all the ligands were stable when attached to HPMA copolymers.

In this communication another photocleavable bond, α-methylphenacyl, was investigated. Copolymers containing side chains terminated in model ligand (Boc-Gly) bound via photocleavable α-methylphenacyl bonds were synthesized. To investigate the photocleavage reaction at the solid–liquid interface, one copolymer was further covalently attached to modified porous silica beads with no spacer

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or with PEO spacers of different lengths (M_r = 1000 and 5000). Copolymers in solution and at the solid–liquid interface were exposed to light resulting in release of the bound ligand. Depending on the type of solvent and length of PEO spacer, differences in the rate of cleavage were obtained. The hydrolytic stability of the α-methylphenacyl bond in solution and at solid–liquid interfaces was also studied.

METHODS

Chemicals

N-(2-hydroxypropyl)methacrylamide was prepared as previously described (mp 69–70°C).[6] 2,2′-Azoisobutyronitrile (AIBN) was recrystallized from ethanol. Porous silica beads, 3-aminopropyl triethoxy silane (APS) coated, 30–40 mesh, 375 Å pore size, were from Fluka. Glutaraldehyde (Glu), 25% solution in water, E.M. Grade was supplied by Polysciences. Diamines $NH_2-PEO_{1000}-NH_2$ and $NH_2-PEO_{5000}-NH_2$ were a kind gift from Dr. S. Nagaoka (Toray Industries, Inc., Kanagawa, Japan). *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and *N*-(3-aminopropyl)methacrylamide hydrochloride were from Pharmacia and Kodak, respectively. Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and aniline were purified by distillation under reduced pressure. Other solvents used were freshly distilled. [^{3}H]-glycine (Amersham) was used without further purification.

Synthesis

Methacrylanilide (1)

Methacrylanilide was prepared according to Ref. 7. The product was crystallized from ethanol, mp 84–85°C (lit.,[7] mp 84–85°C).

ANAL. Calcd for $C_{10}H_{11}NO$ (161.0): C, 74.53%; H, 6.83%; N, 8.69%. Found: C, 74.46%; H, 6.90%; N, 8.60%.

N-[4-(2-Chloropropionyl)phenyl]methacrylamide (2)

Chloropropionyl chloride (66 g, 0.52 mol) was added into the dichloromethane solution (150 mL) of aluminum chloride (84.3 g, 0.63 mol). The reaction proceeded for 10 min under stirring and the solution became dark red. A solution of methacrylanilide (**1**) (30 g, 0.186 mol) and a small amount of hydroquinone in dichloromethane (75 mL) were slowly dropped into the dark red solution. The reaction solution was heated to boiling for 1 h and then hydrolyzed with ice-cold water for 15 min. The dichloromethane layer was collected, dried with magnesium sulfate, and purified with active carbon. The powder residue obtained after removing the dichloromethane was recrystallized twice from acetone/petroleum ether. Yield: 20.5 g, mp 119–120°C.

ANAL. Calcd for $C_{13}H_{14}NClO_2$ (251.5): C, 62.03%; H, 5.57%; N, 5.57%; Cl, 14.12%. Found: C, 61.60%; H, 5.57%; N, 5.36%; Cl, 14.48.

Cesium Salt of [^{3}H]-Boc-Gly (3)

(a) [^{3}H]-Boc-Gly: A mixture of 1.125 g cold glycine (15 mmol) and 1 mCi of [^{3}H]-glycine in 1 mL aqueous solution (specific activity: 19 Ci/mmol) were dissolved in a mixture of dioxane and water (45 mL, vol ratio 2 : 1) and 15 mL of 1*N* NaOH. A dioxane solution of di-*tert*-butyl dicarbonate (3.6 g, 15 mmol) was added into the precooled mixture (4°C). The reaction proceeded under stirring 30 min at 4°C and another 30 min at room temperature. Dioxane and water were rotoevaporated. The residue was dissolved in water and the unreacted di-*tert*-butyl dicarbonate was extracted with ether. The aqueous portion was collected and acidified to pH 2–3 with saturated potassium bisulfate aqueous solution. The crude product ([^{3}H]-Boc-Gly) was extracted twice with ethyl acetate and dried overnight with magnesium sulfate. The next day the magnesium sulfate was filtered off and the dry product (yield: 1.7 g, mp 87°C) was collected by evaporating ethyl acetate.

(b) Cesium salt of [^{3}H]-Boc-Gly: [^{3}H]-Boc-Gly was further reacted with 20% aqueous cesium carbonate (2.94 g, 9 mmol) for 2.5 h. The cesium salt of [^{3}H]-Boc-Gly (**3**) was obtained by removing the water and drying under reduced pressure.

N-{4-[2-(N-Tertbutoxycarbonylglycyloxy)-propionyl]phenyl}methacrylamide (4)

The suspension of **3** (9.8 mmol) in 11.4 mL DMF was added to the DMF solution (1.9 ml) of **2** (0.98 g, 3.9 mmol) with a hydroquinone inhibitor (mole ratio of **2** and **3** = 1 : 2.5). The reaction proceeded for 24 h under stirring at room temperature. A small amount of undissolved powder was filtered off and DMF removed by rotary evaporator. The yellow oil obtained was dissolved in a large amount of ether and the unreacted portion of **3** was extracted with water. The ether portion was collected and put in a freezer for crystallization. The radioactivity of **4** was measured as 0.162 μCi/mg. Yield: 0.36 g, mp 149–150°C. A cold **4** which was sent for elemental anal-

ysis was synthesized by the same method, its elemental analysis was as follows:

ANAL. Calcd for $C_{20}H_{26}N_2O_6$ (390.0): C, 61.54%; H, 6.70%; N, 7.18%. Found: C, 61.69%; H, 6.96%; N, 7.16%.

Copolymerization

Copolymer **5** was prepared by radical precipitation copolymerization of HPMA and **4** (mole ratio 97 : 3) in acetone (12.5 wt % of monomers; 0.6 wt % of AIBN as initiator) at 50°C as previously described.[3,8] The content of monomeric units of **4** in copolymer **5** was calculated to be 2.5 mol % using radioactivity measurement. To perform GPC analysis, a cold copolymer **5** was prepared by the same procedure. The weight- and number-average molecular weights of copolymer **5** (M_w = 100,000; M_w/M_n = 2.8) were estimated from the GPC analysis[9] on a Sepharose 4B/6B (1 : 1) column (90 × 1.6 cm) calibrated with fractions of poly(HPMA) (buffer: 0.5*M* NaCl + 0.05*M* TRIS; pH 8.0).

Copolymer **7** was prepared by radical precipitation copolymerization of HPMA, *N*-(3-aminopropyl)methacrylamide hydrochloride (**6**) and **4** (mole ratio 89.5 : 9 : 1.5) in methanol (12.5 wt % of monomer; 0.6 wt % of AIBN as initiator) as described above. After 24 h at 50°C, the copolymerization was completed and triethylamine was added to transform the hydrochloride into free base. The copolymer solution was precipitated into acetone, reprecipitated, collected, and dried. The content of monomeric units of **4** and **6** was quantified to be 1.4 and 4.7 mol % using radioactivity measurement and the ninhydrin method,[10] respectively. Cold copolymer **7** which was used for molecular-weight determination was prepared the same way. The weight- and number-average molecular weights of copolymer **7** (M_w = 47,000; M_w/M_n = 1.6) were estimated from the GPC analysis.[9]

Modification of Silica Beads

The silica beads were modified according to procedures described in Ref. 11.

Aldehyde Beads:)—APS—GLU—CH=O (9)

One gram of APS coated beads (**8**), containing 14 nmol NH_2/mg, were reacted with 20 mL 2.5% glutaraldehyde (GLU) in borate buffer, pH 8.6 (batch A), or carbonate buffer, pH 9.5 (batch B). The reaction mixture was gently shaken overnight at room temperature, and then washed many times with water, ethanol, ether and dried. No NH_2 groups were detectable on beads by a qualitative test using the ninhydrin method.[10]

Amino Beads:)—APS—GLU—PEO_{1000}—NH_2 (10),)—APS—GLU—PEO_{5000}—NH_2 (11)

One gram of aldehyde beads **9** (batches A and B) were reacted with a large excess of the respective diamine solution: 5 mL 20% NH_2—PEO_{1000}—NH_2 or 4 mL 50% NH_2—PEO_{5000}—NH_2 in sodium carbonate/bicarbonate buffer pH 9.0. Beads were gently shaken overnight and washed thoroughly as described above. The azomethine bond formed was reduced by reaction with 80 mg $NaCNBH_3$ in 3 mL sodium carbonate/bicarbonate buffer pH 9.5. After proper washing and drying the content of NH_2 was determined as described below. No aldehyde groups on beads were detectable after diamine treatment by the reaction with dinitrophenylhydrazine.[12]

Modification of PEO Spacer-Containing Beads: Preparation of)—APS—GLU—PEO_{1000}—GLU—CH=O (12),)—APS—GLU—PEO_{5000}—GLU—CH=O (13)

One gram of NH_2 groups containing beads **10** and **11** (batches A and B), respectively, were treated with 20 mL 2.5% glutaraldehyde by the same procedure as described above. The remaining unreacted NH_2 groups after glutaraldehyde treatment were quantified as described below.

Copolymer-Derivatized Silica Beads:)—APS—GLU—CH_2—NH—Copolymer 7 (14),)—APS—GLU—PEO_{1000}—GLU—CH_2—NH—Copolymer 7 (15), and)—APS—GLU—PEO_{5000}—GLU—CH_2—NH—Copolymer 7 (16)

Five hundred fifty milligrams of beads **9**, **12**, and **13** (batch A), respectively, were treated with excess of the copolymer **7** (500 mg) in DMSO. The suspension was gently shaken overnight at room temperature, and then washed many times with ethanol and methanol. The azomethine bond was further reduced by reaction with 50 mg $NaCNBH_3$ in phosphate/citric buffer, pH 4.5. After proper washing and drying the binding efficiency was quantified as described below.

Nonspecific Adsorption of Copolymer 7 to Silica Beads

To estimate the extent of the nonspecific adsorption of copolymer **7**, beads **8**, **10**, and **11** were incubated with copolymer **7** as described above. Beads with physically adsorbed copolymer **7** were numbered **17**, **18**, and **19**, respectively (see Table II).

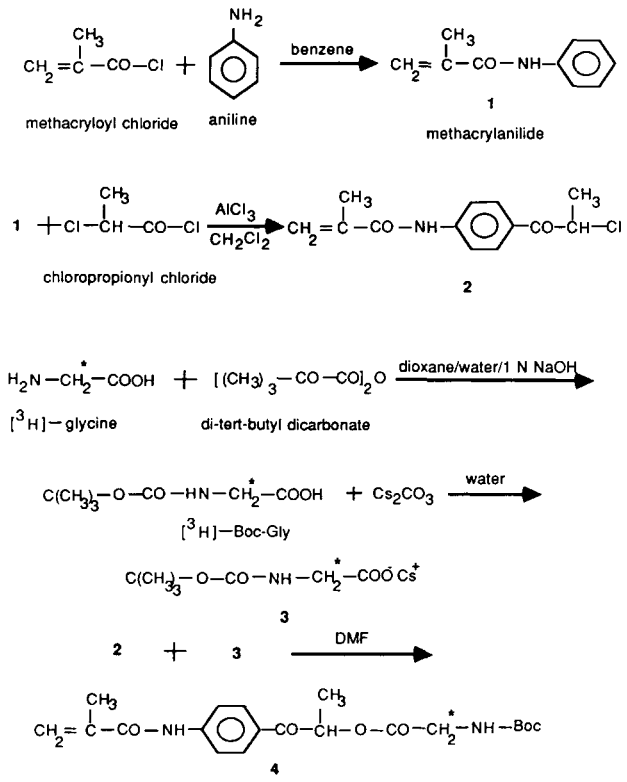


Figure 1 Synthesis of monomers **1–4.**

Analysis of Beads

Determination of NH_2 Groups. The concentration of amino groups on the beads was determined using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP).[13] The beads were treated with an excess of SPDP in the presence of a small amount of 4-dimethylaminopyridine (catalyst). The unreacted reagent was removed by washing, followed by reduction of acylated beads by dithiothreitol (DTT). This results in the release of pyridine-2-thione, whose concentration was determined spectrophotometrically at 343 nm (ϵ = 8080 L/mol cm).

Surface Area of Beads. The specific surface of original APS coated beads was 33.4 m^2/g. All modified beads (batch A) had specific surface in the range from 29 to 31 m^2/g.[11]

Radioactivity Measurement

All ^3H radioactivity measurements were performed with a Beckman LS1801 scintillation counter using biodegradable scintillation cocktail (National Diagnostic, NJ) for photocleavage studies in solution and Ready-Gel cocktail (Beckman) for studies at the solid–liquid interface.

Determination of Radioactivity on Silica Beads

Twenty to thirty milligrams of beads and 0.5 mL 6*N* HCl were put into glass ampoules. The ampoules were sealed and heated at 110°C for 12 h. After cooling the ampoules, the aliquots were transferred to scintillation vials, diluted, neutralized with 6*N* NaOH, and the radioactivity measurement performed.

Hydrolytic Stability

Hydrolytic Stability of Copolymer 5 in Solution

Copolymer **5** was incubated with buffers having different pH's in test tubes at room temperature or 37°C in the dark. After incubation at various time intervals, the copolymer solution (1 wt %) was applied to a PD-10 column (Pharmacia) equilibrated with the buffer. Fractions corresponding to high- and low-molecular-weight substances were pooled and their radioactivities determined. The structure of Boc-Gly released by hydrolysis was verified by TLC (Silica gel 60 F254 from EM Laboratories, Inc.) using 1-butanol/acetic acid/water (vol. ratio 3 : 1 : 1).[14]

Hydrolytic Stability of Copolymer 7-Containing Silica Beads 14

Silica beads (30 mg **14**) and 0.4 ml pH 4.5 citrate/phosphate or pH 7.2 phosphate buffers were incubated in 37°C. The radioactivity of the supernatant was measured at time intervals always before adding fresh buffer. The hydrolytic stability against 50% trifluoroacetic acid (TFA)/CH_2Cl_2 was performed at room temperature similarly as described above.

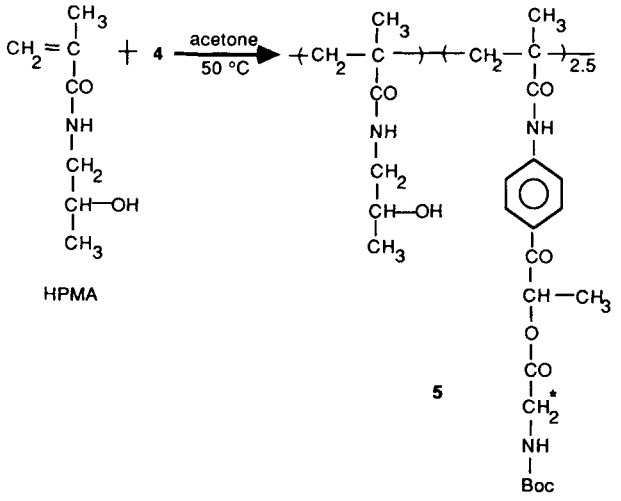


Figure 2 Synthesis of copolymer **5.**

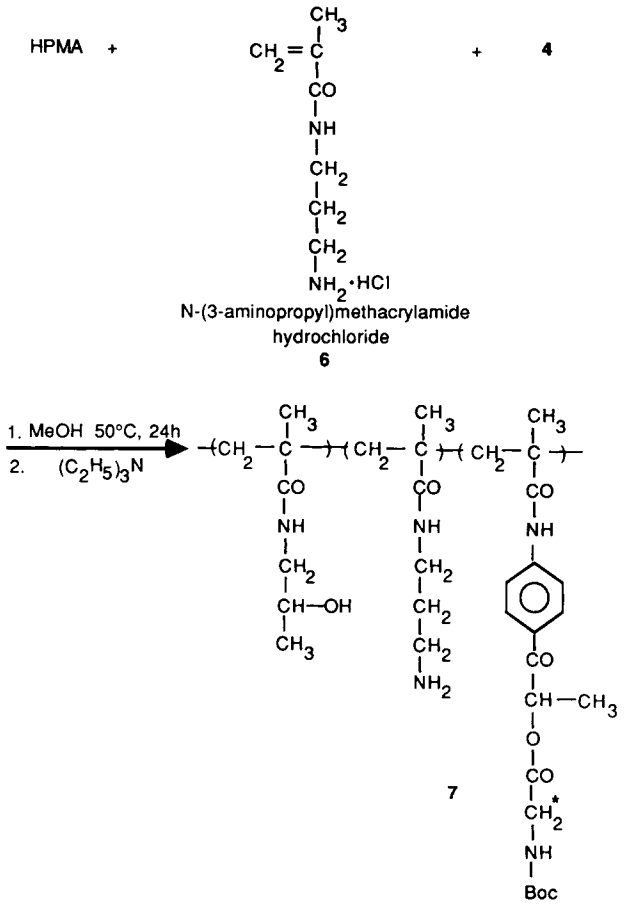


Figure 3 Synthesis of copolymer **7**.

Photocleavage

Photocleavage in Solution

Copolymer **5** was dissolved in methanol or ethanol to form a 1 wt % solution and then deaerated with nitrogen for 30 min. The copolymer solution was irradiated in a quartz cuvette (d = 1 cm) with a LH 150 mercury lamp (200 W, Schoeffel Instr. Co.) with maximal emission at 360 nm for various time periods (intensity: 8 mW/cm^2 at 365 nm; a focusing lens was placed between lamp and the sample). After irradiation, the solution was applied to Sephadex LH-20 column (28 × 1 cm) and the radioactivities of the individual fractions were measured. The decrease of radioactivity in the copolymer reflected the amount of Boc-Gly released (analysis was done in triplicate). The structure of Boc-Gly released was qualitatively verified by TLC.[14]

Photocleavage at the Solid–Liquid Interface

Thirty milligrams of the copolymer **7**-derivatized silica beads (**14**, **15**, and **16**) and 0.5 mL dehydrated ethanol were poured into UV quartz cylindrical cell with Teflon stopper. The cell was attached to a rotator (Clas-Col Co.) with speed 60 rpm, so that during irradiation a centrifugal force gently agitated beads to ensure the same probability of light access to every part of the surface. The cell was irradiated with a LH 150 mercury lamp (200 W, Schoeffel Instr. Co). The emission was first focused by a fused silica lens, then reflected by a dichroic-coating mirror (Oriel Corp.) which reflects only the wavelengths between 260 and 340 nm (the major intense emission peaks are 296 and 312 nm). At chosen time intervals, the supernatant was withdrawn for radioactivity measurement. The increase of radioactivity in the supernatant reflected the amount of photoreleased ligand. Each experiment was performed twice and average data were plotted (the differences between these two sets of data were within 3%). After irradiation, the remaining radioactivities on the silica beads were determined as described above. The mass balance of radioactivity between supernatant and silica bead surfaces were consistent throughout the experiments. Controls were kept in the dark. The Boc-Gly released was qualitatively verified by TLC as described above.[14]

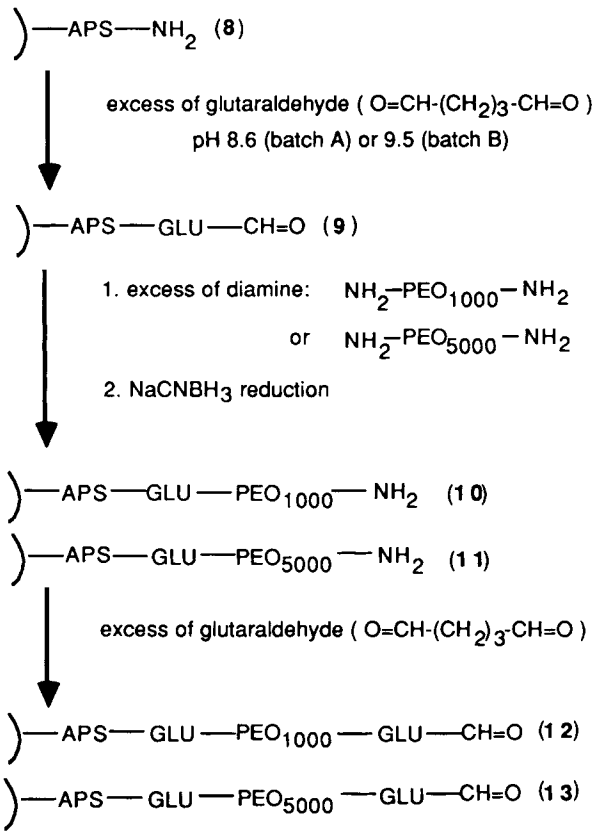


Figure 4 Modification of porous silica beads.

Table I The Content of NH_2 Groups on Silica Beads 8–13

Silica Bead No.	Schematical Structures	Content of Amino Group (NH_2) (nmol/mg) Batch A[a]	Batch B[b]
8	)—APS—NH_2	14	14
9	)—APS—GLU—CH=O	0	0
10	)—APS—GLU—PEO_{1000}—NH_2	9	7
11	)—APS—GLU—PEO_{5000}—NH_2	10	7
12	)—APS—GLU—PEO_{1000}—GLU—CH=O	1	1
13	)—APS—GLU—PEO_{5000}—GLU—CH=O	2	1

[a] These beads were prepared by reacting with glutaraldehyde at pH 8.6.
[b] These beads were prepared by reacting with glutaraldehyde at pH 9.5.

RESULTS

Synthesis of Monomers and Copolymers

The monomer containing Boc-Gly bound via α-methylphenacyl group was synthesized by a series of reactions. By the reaction of methacryloyl chloride with aniline in benzene, methacrylanilide **1** was obtained,[7] which was further reacted with chloropropionyl chloride to yield **2.** An excess amount of the cesium salt of [^{3}H]-*N-tert*-butoxycarbonylglycine (**3**) were then allowed to react with **2** in dimethylformamide to obtain the final product **4** (Fig. 1). The photocleavable copolymer **5** was prepared by copolymerization of HPMA and **4** in acetone (Fig. 2). In order to attach the copolymer to modified silica beads, *N*-(3-aminopropyl)methacrylamide hydrochloride (**6**), a comonomer containing free NH_2 groups, was incorporated into copolymer **7.** The latter was synthesized by copolymerization of HPMA, **4** and **6** in methanol (Fig. 3).

Modification of Silica Beads

The modification of porous silica beads is schematically shown in Figure 4.[11] The APS-coated beads **8** contained 14 nmol/mg of amino groups. After reaction with a large excess of glutaraldehyde, practically all amino groups were converted to aldehyde groups (beads **9**). The latter were modified with a large excess of diamines, NH_2—PEO_{1000}—NH_2 and NH_2—PEO_{5000}—NH_2. The resulting beads **10** and **11** contained 7–10 nmol/mg of terminal amino groups. Due to the incomplete reaction of beads **10** and **11** with excess of glutaraldehyde, the beads **12** and **13** still contained less than 2 nmol/mg of unreacted NH_2 groups on surfaces (Table I). To evaluate the influence of pH on the binding reactions, two procedures were chosen: In batch A the reaction with glutaraldehyde was performed at pH 8.6, whereas in the batch B it was performed at pH 9.5. The content of NH_2 groups on beads **10** and **11** of batch A was 2–3 nmol higher than in batch B (Table I). The surface area of the beads (batch A) did not show any dramatic change during these polymer-analogous reactions.[11]

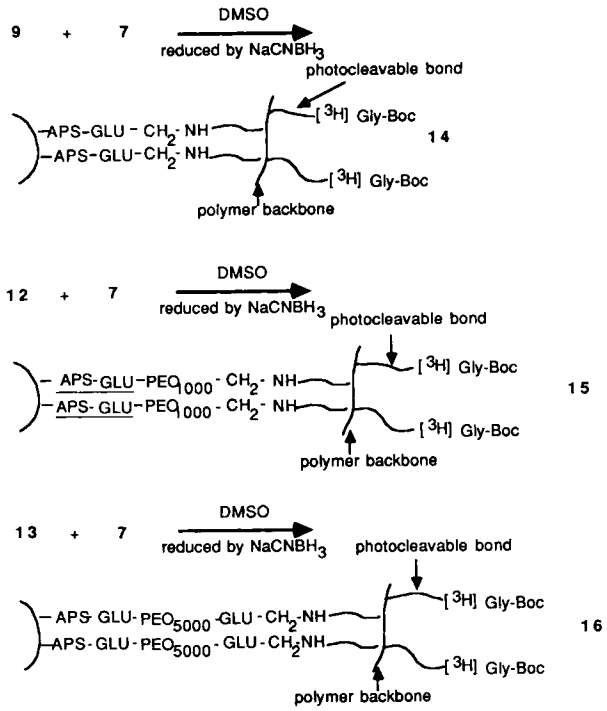


Figure 5 Synthesis and schematic structures of silica beads **14, 15,** and **16.**

Table II The Radioactivities (DPM) and Amounts of Copolymer 7 Bound on Modified Silica Beads 14–19

Beads No.	Radioactivity of Bound Copolymer 7 (DPM/50 mg beads)	Amount of Bound Copolymer 7 (mg/50 mg beads)	Amount of Bound Copolymer 7 (mg/m^2)[a]
14[b]	24,900	1.81	1.21
15[b]	15,400	1.12	0.75
16[b]	10,300	0.75	0.50
17[c]	5700	0.4	0.28
18[c]	1100	0.08	0.06
19[c]	300	0.02	0.01

[a] The specific surface area of 30 m^2/g of bead was used as a basis for calculation.
[b] Covalent attachment to beads **9**, **12**, and **13**, respectively.
[c] Nonspecific adsorption on beads **8**, **10**, and **11**, respectively.

Copolymer-Derivatized Silica Beads

Silica beads **14**, **15**, and **16**, containing copolymer **7**, were synthesized by treating silica beads **9**, **12**, and **13**, respectively, with copolymer **7**. Their schematic structures are shown in Figure 5. The amounts of copolymer **7** on silica beads **14–16** were quantified by hydrolyzing the beads with 6*N* HCl at 120°C for 12 h, followed by measurement of the radioactivities of the supernatants. The radioactivities and amounts of copolymer bound on silica **14–16** are shown in Table II. To estimate the nonspecific adsorption of copolymer on silica surface, the radioactivities of physically adsorbed copolymer **7** on beads **8**, **10**, and **11** were included for comparison. The results suggest that the amounts of covalently bound and of nonspecific adsorption of HPMA copolymer decreased as the length of PEO spacer increased.

Hydrolytic Stability of α-Methylphenacyl Bond

Hydrolytic Stability of α-Methylphenacyl Bond in Solution in the Dark

Table III shows the degree of hydrolysis of α-methylphenacyl bond in copolymer **5** at different pH's. As expected the ester bond (see structure of copolymer **5** in Fig. 2) between [^{3}H]-Boc-glycine and the polymeric backbone was more stable toward hydrolysis under acidic rather than alkaline conditions, yet the extreme instability in pH 7.2 buffer (close to pH of human blood) was somewhat surprising. Copolymer **5** was almost completely hydrolyzed within minutes in more alkaline conditions, i.e., at pH's 9.5 and 10.7. The time course of copolymer **5** hydrolysis at pH 7.0 and 7.2 at 37°C is shown in Figure 6. At pH 7.2, 90% hydrolysis occurred within 10 h.

Hydrolytic Stability of α-Methylphenacyl Bond at the Solid–Liquid Interface in the Dark

The hydrolytic stability of the α-methylphenacyl bond at the solid–liquid interface is shown in Figure 7. Again, as expected, the bond is stable in pH 4.5 and unstable in pH 7.2 at 37°C. However, the rate of hydrolysis at pH 7.2 at the solid–liquid interface was considerably slower when compared to the hydrolysis in solution (compare curve A, Fig. 6 with curve A, Fig. 7; 90% of bonds were hydrolyzed in solution within 10 h, whereas only 30% at the surface). It appears that the surface has a stabilizing effect against hydrolysis. This anchoring bond was completely stable at the solid–liquid interface in 50% TFA/CH_2Cl_2, but it was labile toward hydrolysis in solution (data not shown). This was consistent with previously published data.[15]

Photocleavage

Photocleavage in Solution

The photocleavage of copolymer **5** was carried out in ethanolic and methanolic solutions in a quartz cuvette under nitrogen atmosphere. During irradiation, the Boc-Gly bound via α-methylphenacyl group was released from the polymeric support. The structure of the released ligand (Boc-Gly) was confirmed by TLC.[14] Figure 8 shows the rates of photocleavage release in methanolic and ethanolic solutions (the photocleavage rate of Boc-Gly from HPMA copolymer containing 2-nitrobenzyl group in methanol solution is included for comparison[3]). The rate of photocleavage of the HPMA copolymer containing the

Table III Hydrolytic Stabilities of Copolymer 5 at 37°C at Different pH[a]

pH	% of Hydrolysis
2[b]	< 5
4.5[b]	< 5
7.2[b]	96
9.5	Almost completely hydrolyzed within minutes
10.7	Almost completely hydrolyzed within minutes

[a] Buffers: pH 2—hydrochloric acid/potassium chloride buffer; pH 4.5—citrate/phosphate buffer; pH 7.2—phosphate buffer; pH 9.5 and 10.7—carbonate/bicarbonate buffer.
[b] Incubation for 10 h.

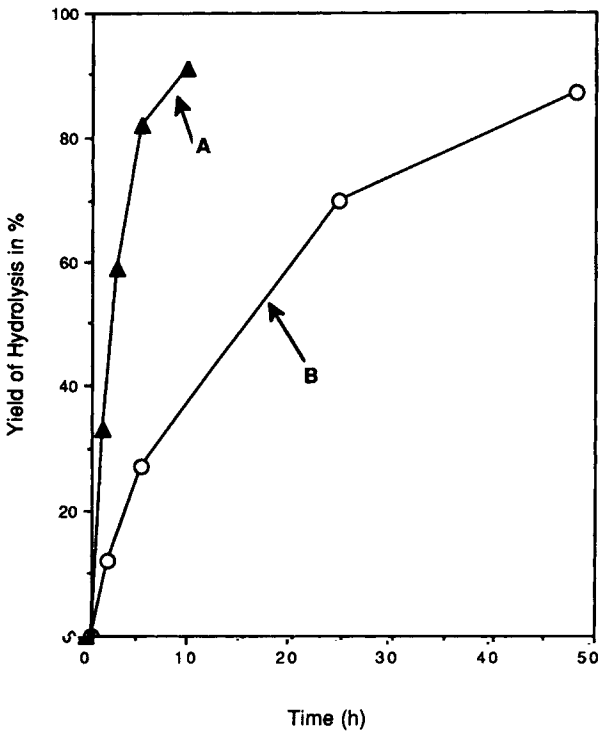


Figure 6 Time-dependent hydrolysis of α-methylphenacyl bonds in solution: (A) in pH 7.2 phosphate buffer at 37°C; (B) in pH 7.0 phosphate buffer at room temperature.

α-methylphenacyl group in ethanol was faster than in methanol (Fig. 8). The rate of the 2-nitrobenzyl group cleavage in methanol was intermediate between the other two rates.

Photocleavage at Solid–Liquid Interface

The rates of photocleavage of copolymer-derivatized silica beads **14**, **15**, and **16** in ethanol are shown in Figure 9. In all three silica beads the patterns of photocleavage were similar. The half-lives of photolysis of **14**, **15**, and **16** were approximately 12, 8, and 7 h, respectively. Photolysis for all three bead systems proceeded rapidly in the first 2 h, with more than 20% of ligand release in the first hour. The rate of photolysis increased with an increase in the length of the PEO spacer.

DISCUSSION

The ultimate aim of this series of papers is to determine the feasibility of the development of a ligand delivery system which is remotely controlled by light. Basically the ligand, e.g., fluorescein-labeled or radioactive antigen, is coupled via a photolabile bond to a polymer matrix. Irradiation of light of the proper intensity and wavelength results in bond breakage, providing a released ligand, which then competes with circulating ligand for the antibody binding sites on the sensor surface.[2] In this way, an immunosensor that is remote and continuous with sensitivity and specificity can be developed.[16]

Sensors with different geometries can be developed. Their design may be based on two optical fibers, two-plate geometry or capillary-fill devices.[17] Regardless of geometry, fluoroimmunosensors are based on the competitive binding of antigen (or hapten) and an (optically) released fluorescently labeled antigen to an immobilized antibody. The detection of the antigen–antibody reaction at the solid–liquid interface can be performed using total internal reflection fluorescence.[2]

This study, however, focuses on the possibility to release a model ligand (Boc-Gly) bound via α-methylphenacyl bonds to a polymeric carrier under optical control both in solution and at a solid–liquid interface.

Type of Bond between the Carrier and Ligand

There are many types of photocleavable bonds used in organic synthesis.[18] Among these, the α-methyl-

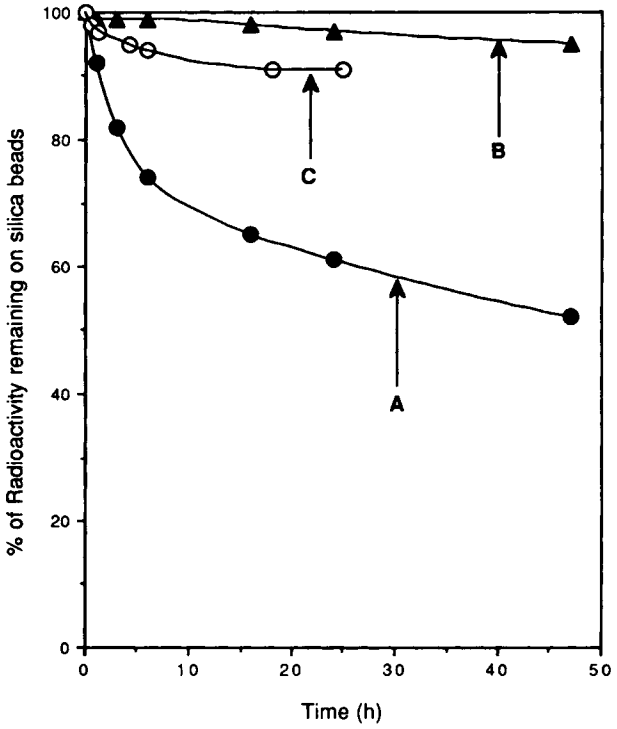
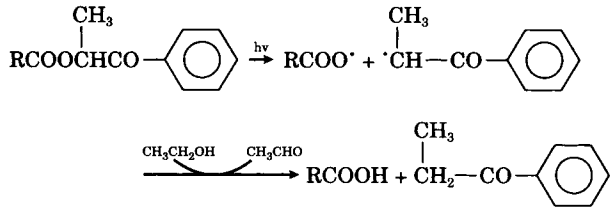


Figure 7 Stability of α-methylphenacyl bonds at solid–liquid interface: (A) in pH 7.2 phosphate buffer at 37°C; (B) in pH 4.5 citrate/phosphate buffer at 37°C; (C) in 50% TFA/CH_2Cl_2 at room temperature.

phenacyl ester bond[19] is of particular interest since it can be readily introduced into polymer matrices.[20] This bond was used as an anchoring linkage between peptide chains and polymer supports in solid and liquid phase peptide synthesis. For example, it was shown that the photolytic rate of tetrapeptide release from a styrene–divinylbenzene copolymer was faster when bound via an α-methylphenacyl bond than when bound via the more frequently used 2-nitrobenzyl bond.[15]

The α-methylphenacyl bond has low-lying excited states because of the interaction of the electrons between the carbonyl group and the phenyl ring. Such interactions render this group photolytically cleavable.[19] The photolytic mechanism is considered to be a simple radical scission of the carbon–oxygen bond.[19] It should be pointed out that the rate of photolytic cleavage of the α-methylphenacyl group is solvent-dependent. The solvents used should behave as hydrogen donors to accelerate the reaction. The photolytic mechanism is shown below[19]:



Synthesis

The copolymer **5** used for the photocleavage study in the solution was prepared by copolymerization of HPMA with comonomer **4** containing Boc-Gly bound via an α-methylphenacyl group. To study the photocleavage at a solid–liquid interface a water soluble HPMA copolymer was synthesized which contained additional side chains terminated in NH_2 groups. APS modified porous silica (beads **8**) was used as a solid support. Primary amino groups on its surface (14 nmol/mg) were converted to aldehyde groups by reaction with an excess of glutaraldehyde (beads **9**). Two pH's were used for this reaction, pH 8.6 and 9.5. The resulting beads **9** did not contain any remaining NH_2 groups indicating total conversion. The subsequent reaction was the attachment of α,ω-diaminopoly(ethylene oxide)s. The resulting beads **10** and **11** had a higher content of NH_2 groups when beads **9** were prepared at pH 8.6 (batch A) than those prepared at pH 9.5 (batch B). This indicated that in the latter case part of the aldehyde groups were consumed by polymerization at the surface resulting from aldol condensation.[21–23] Consequently, only silica beads prepared at pH 8.6 (batch A) were used for further investigation.

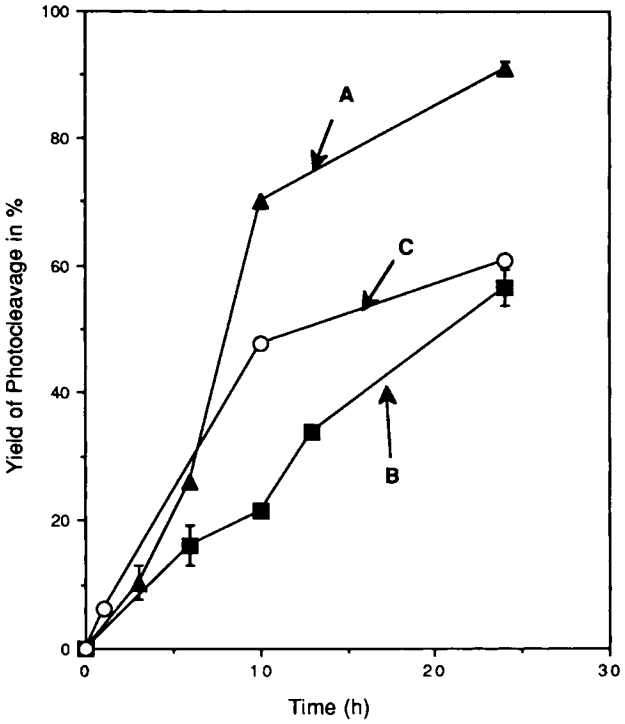


Figure 8 Cleavage of Boc-Gly obtained by photolysis of HPMA copolymers: (A) copolymer 5 in ethanol; (B) copolymer 5 in methanol; (C) HPMA copolymer containing 2-nitrobenzyl group in methanol.[3]

The subsequent reaction (Fig. 4) was again the attachment of glutaraldehyde to beads **10** and **11**. This reaction was not fully complete, resulting beads **12** and **13** contained a small amount of residual NH_2 groups (Table I). The ligand was introduced at the surface by binding of copolymer **7** which contained side chains terminated in NH_2 groups to beads **9**, **12**, and **13** containing terminal aldehyde groups (Table II).

The PEO spacer was chosen for this study because it exerts a unique protein-resistant property at solid–liquid interfaces, probably due to its low interfacial free energy with water, unique solution properties and molecular conformation in aqueous solution, hydrophilicity, high surface mobility, and steric stabilization effects.[11,24–26] The results obtained (Table II) demonstrate that the PEO spacer could reduce the nonspecific adsorption of HPMA copolymer **7** on modified silica surfaces. The degree of nonspecific adsorption of HPMA copolymers on silica surface may depend on several factors, e.g., on the density of PEO chains on the surface and on

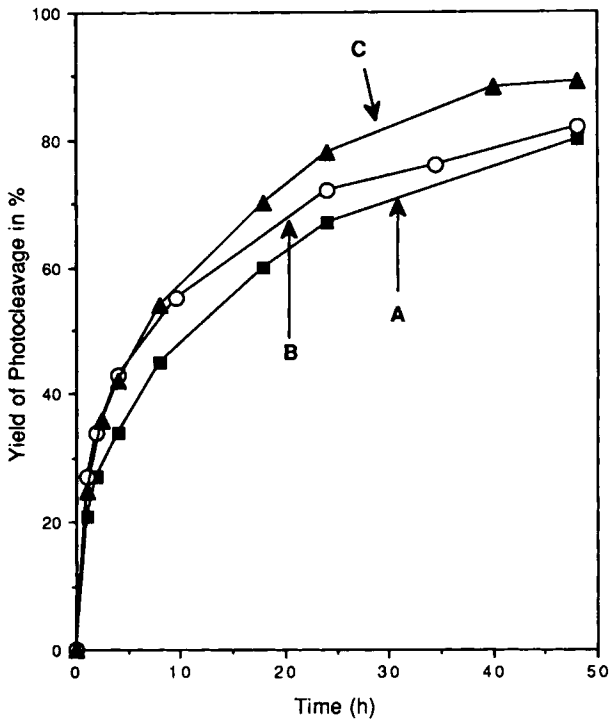


Figure 9 Photocleavage of copolymer **7**-containing silica beads **14** (A), **15** (B), and **16** (C) in ethanol.

their molecular weight. Because of the similar specific surface areas of beads studied,[11] similar content of amino groups at the surface of silica beads **10** and **11** (Table I), and the low possibility for loop formation during PEO attachment (under experimental conditions used), the packing density of PEO chains on surfaces of beads **10** and **11** should be similar. Consequently, the decrease of nonspecific adsorption of copolymer **7**, with increasing length of PEO spacers observed, reflects the molecular weight influence. The amount of covalently bound copolymer **7** on the silica beads also decreased as the length of PEO spacer increased. This is probably due to the lower concentration of free aldehyde groups on silica beads **12** and **13** than on silica beads **9**.

Hydrolytic Stability

During synthesis, the lability of the α-methylphenacyl bond toward acidic conditions was observed in solution. Rapid loss of radioactivity from copolymer backbone (**5**) occurred in 30 wt % HCl/MeOH, 25 and 50% trifluoroacetic acid (TFA)/CH_2Cl_2 at room temperature. The rate of hydrolysis in aqueous solution increased with increasing pH. The increased susceptibility to hydrolysis in alkaline pH's may be due to the polar effect of the carbonyl near the phenyl group[27] and the formation of hydrogen bonds between the acyl oxygen of the ester bond and the hydrogen of amide bond.

On the other hand, when the α-methylphenacyl group was bound at the solid–liquid interface, the surface offered surface-stabilizing effect to improve the resistance to hydrolysis. Wang showed that the α-methylphenacyl ester bond in Z—Lys(Z)—Phe—Phe—Gly—OCH(CH_3)—CO—C_6H_4-resin (crosslinked polystyrene beads) was stable against 50% TFA/CH_2Cl_2,[15] and we have observed the same. The surface stabilizing effect is not unexpected. Went et al.[28] studied the hydrolysis of polyacrylamide attached to polystyrene latex particles. The rate of hydrolysis of the former was significantly lower when compared to the rate of polyacrylamide hydrolysis in solution. Apparently, conformational changes of an attached polymer are restricted so that hydrolysis in the regions far from surface is more likely. Consequently, in alkaline solution, due to the concentration of charged carboxylic groups in the peripheral zones of the attached polymer, the repulsion of incoming hydroxyl ions is enhanced, leading to a reduced rate of hydrolysis.[28]

Photocleavage

The photocleavage of copolymer **5** in solution is shown on Figure 8. In both solvents used, methanol and ethanol, the cleavage was fast and approximately linear to 60% of Boc-Gly released. The rate of photocleavage was faster in ethanol than in methanol. During the photocleavage of α-methylphenacyl bond the solvent serves as a hydrogen donor. The differences in the rate of photocleavage can be attributed to the fact that ethanol is a better hydrogen donor. This is consistent with the observation that the rate of photofading of aqueous alcohol solutions of dyes increases with increasing susceptibility of the alcohol towards hydrogen atom abstraction, viz. methanol < ethanol,[29] as well as with the higher chain transfer constant to ethanol than to methanol during polymerization of butyl acrylate.[30]

At the solid–liquid interface the photorelease of Boc-Gly from beads **14**, **15**, and **16** was evaluated in ethanol (Fig. 9). At the onset of photolysis the rate of release was the same. As photolysis continued, the rate of Boc-Gly release was faster for the beads with PEO spacers (beads **15** and **16**) compared to the bead without spacer (bead **14**). The results were similar to the photolytic data obtained with 2-nitrobenzyl photocleavable bonds.[31] The short interval of fast and nearly linear ligand release

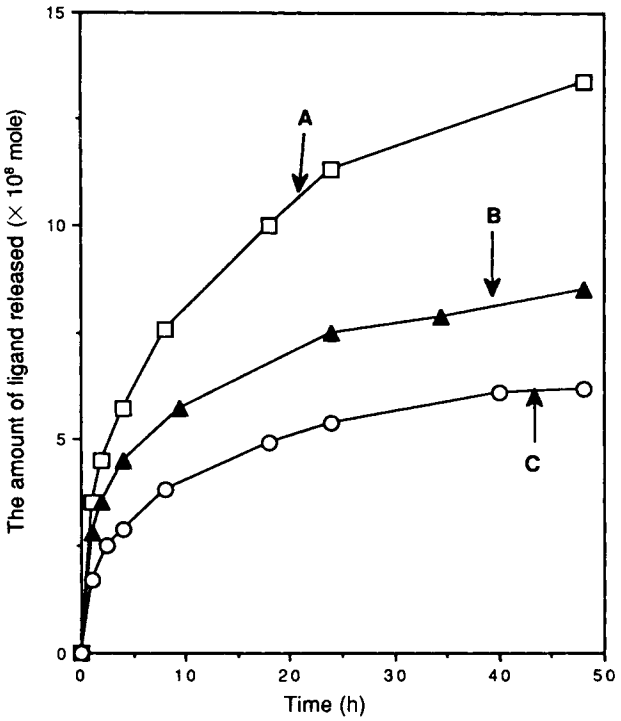


Figure 10 The amount of photoreleased ligand from 50 mg of silica beads **14** (A), **15** (B), and **16** (C) in ethanol.

would be most suitable for ligand delivery applications.

The differences in photolytic rates between **14**, **15**, and **16** could be due to the change of microenvironment at the solid–liquid interface, resulting in the change in quantum yield of this photolytic reaction. One possible explanation lies in the flexibility of the PEO spacer.[32,33] The higher mobility of the PEO spacer might make the surface-bound polymer more flexible. Under the photolytic condition, i.e., a nonpolarized light source (medium-pressure mercury lamp), the more mobile polymer would have a higher efficiency of light adsorption and result in a higher photolytic rate.

For developing an optical controlled ligand delivery system several factors, e.g., the amount of photocleavable ligand chemically bound on the surface, nonspecific adsorption, and the rate of photolysis, should be optimized. The capacity of repetitious usage of this system is dependent on the amount of ligand chemically bound on the surface. Nonspecific adsorption can influence the long-term stability of this ligand delivery system due to exposure to different test solutions and body fluids. This problem can be minimized using PEO spacers.[11] The rate of photolysis will influence the operation speed of this delivery system. Figure 10 shows the amount of ligand released vs. time for 50 mg of silica beads **14**, **15**, and **16** in ethanol. It showed that the total amount of ligand released decreased with an increase of the length of the PEO spacer, because the amount of ligand which was chemically bound on silica surface decreased (Table II) with the increase of the length of PEO spacer. For silica beads **14**, the amount of ligand loaded on silica surface was the largest, yet the rate of photolysis was lowest and the ability to prevent nonspecific adsorption was poorest. On the other hand, although silica **16** showed the highest rate of photolysis and good capacity for preventing nonspecific adsorption, the amount of ligand chemically bound on surface was the lowest. A simple way to increase load of ligand on surface would be to incorporate a higher mol % of photoreleasable ligand into the copolymer (in this model study, the copolymer **7** contained only 1.4 mol % of side chains terminated with photocleavable ligand).

From the experiments on the photocleavage of α-methylphenacyl bond in solution and at the solid–liquid interface, it may be concluded that in solution the photolysis rate was greater in ethanol than in methanol, perhaps due to the higher hydrogen donor capacity of ethanol. At the solid–liquid interface, the rate of photorelease increased as the length of PEO spacer increased. Although the instability of this bond toward alkaline hydrolysis would limit its application for sensors used *in situ*, it still has the potential to be applied as a ligand delivery system in organic solvents. From another point of view the alkaline hydrolytic property could also be used as a tool for pH-dependent ligand delivery, e.g., in stimuli-sensitive delivery systems, such as hydrogels or liposomes.[34]

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