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IgG-Polydiacetylene Sol-Gel Nanocomposites as Solid-State Chromatic Biosensors

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Materials. Heneicosa-2,4-diynoic acid (21:2$\Delta^{2a,4a}$, 2,4-HCDA), docosa-5,7-diynoic acid (23:2$\Delta^{2a,4a}$, 5,7-DCDA), tricosa-2,4-diynoic acid (22:2$\Delta^{5a,7a}$, 2,4-TRCDA), tricosa-5,7-diynoic acid (23:2$\Delta^{5a,7a}$, 5,7-TRCDA), tetracosa-5,7-diynoic acid (24:2$\Delta^{5a,7a}$, 5,7-TCDA) and pentacosa-10,12-diynoic acid (25:2$\Delta^{2a,4a}$, 10,12-PCDA) were purchased from Farchan Labs (Gainsville, FL, USA), GFS Chemicals (Powell, OH, USA) and Lancaster Synthesis (Lancashire, UK), and were purified by recrystallization from petroleum ether and 3:1 or 9:1 petroleum ether-dichloromethane. Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE), oleoylpalmitoylphosphatidylethanolamine (OPPE), linoleoylpalmitoylphosphatidylethanolamine (LPPE), soybean lecithin (SL), egg lecithin (EL), Mouse anti-Human α-fetoprotein, *E. coli* β-glucuronidase, *E. coli* β-galactosidase, bovine serum albumin (BSA), triphenylphosphine (TPP), tetramethoxysilane (TMOS), 1-(3-dimethylaminopropyl)3-3ethylcarbodimimde hydrochloride (EDC), *N*-sulfosuccinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), *N*, *N*'-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 3-

glycidoxypropyltrimethoxysilane (3-GPTMOS) and glycerol were obtained from Aldrich (Dorset, UK and St. Louis, MO, USA). *N*,*N*-dimethyl-3-aminopropyltrimethoxysilane (DAPSOMe), *N*,*N'*-bis(3-trimethoxysilylpropyl)ethylenediamine (BSPEOMe), 3-aminopropyltrimethoxysilane (3-APTEOS) and 3-ureidopropyltriethoxysilane (3-UPTEOS) were obtained from United Chemical Technologies, PA, USA. Rabbit anti-*E. coli* β-glucuronidase IgG, rabbit anti-*E. coli* β-galactosidase IgG, rabbit anti-bovine serum albumin IgG and rabbit anti-yeast alkaline phosphatase IgG, monoclonal antibodies were purchased from Molecular Probes (Eugene, OR, USA, and Leiden, Netherlands).

Analytical techniques. HPLC was carried out on an Agilent 1100 series HPLC equipped with a UV-VIS diode array detector and ChemStation data acquisition hardware. Analyses of diacetylenic acids and their amides were performed on a 0.46 x 25 cm, 5 μm, Nucleosil C18 column (Phenomenex , USA), eluted with water-acetonitrile (30% to 80% v/v acetonitrile in 15 min, then to 100% v/v acetonitrile in 5 min, hold at 100% v/v acetonitrile for 5 min), with detection at 254 nm. FAB-MS spectra were recorded on a Kratos MS9 instrument equipped with a xenon gun operating at 5-7 kV. Samples were dispersed in glycerol and applied to a copper probe and spectra were recorded at 100 mmu in positive mode, using polyglycerol ions as reference.

Preparation of diacetylenic acid 2'-aminoethylamides. (a) The EDC/NHS method was used for amidation of 2,4-TRCDA, 5,7-TRCDA and 5,7-TCDA: A solution of EDC (2.4 mmol, in 10 mL dichloromethane), followed by a solution of NHS (2.4 mmol, in 5 mL dichloromethane) were added to a solution of diacetylenic acid (2.45 mmol, in 25-30 mL THF), and the mixture

stirred (300 rpm) in the dark at room temperature for 20 h. The reaction mixture was rotary evaporated at 30 ^{O}C, the residue extracted with chloroform (30 mL), the organic layer washed with brine (6 x 20 mL), dried over anhydrous magnesium sulfate, rotary evaporated to dryness, and the residue dissolved in chloroform (50-70 mL). The obtained solution of acetylenic acid NHS ester was added over 20-30 min to a solution of ethylenediamine (4 mmol, in 20 mL chloroform) and the mixture stirred (300 rpm) in the dark at room temperature for 3 h. The reaction mixture was concentrated by rotary evaporation at 30 ^{O}C, and the mixture purified by TLC or flash chromatography on silica (Kieselgel 60, elution with 4:1 or 5:1 chloroform-methanol) to give the desired 2'-aminoethylamide with a yield of 83-91% and purity of 95-99%; (b) The DCC method was employed for 5,7-DCDA and 10,12-PCDA: A solution of DCC (2.4 mmol, in 10 mL dichloromethane) was added to a solution of diacetylenic acid (2.45 mmol, in 20 mL dichloromethane) and the solution stirred (200 rpm) in the dark at room temperature for 1 h. The solution was then added to a solution of ethylenediamine (4 mmol, in 10 mL dichloromethane) over 20 min, and the mixture stirred (300 rpm) in the dark at room temperature for 1 h. The mixture was diluted with petroleum ether (20 mL), the suspension filtered through Celite, rotary evaporated to dryness at 30 ^{O}C, and the residue purified by TLC or flash chromatography on silica (Kieselgel 60, elution with 4:1 or 5:1 chloroform-methanol) to give the desired product with a yield of 88-95% and purity of 96-99%. The identities of the 2'-aminoethylamides were confirmed by positive ion FAB-MS: 2,4-diyn-C_{21}-2'-aminoethylamide (M+H) calcd for $C_{23}H_{41}N_2O$, 361.3219, obsd, 361.3248; 2,4-diyn-C_{23}-2'-aminoethylamide (M+H) calcd for $C_{25}H_{45}N_2O$, 389.3532, obsd, 389.3517; 5,7-diyn-C_{22}-2'-aminoethylamide (M+H) calcd for $C_{24}H_{43}N_2O$, 375.3375, obsd, 375.3541; 5,7-diyn-C_{23}-2'-aminoethylamide

(M+H) calcd for $C_{25}H_{45}N_2O$, 389.3532, obsd, 389.3550; 5,7-diyn-C_{24}-2'-aminoethylamide (M+H) calcd for $C_{26}H_{47}N_2O$, 403.3688, obsd, 403.3709.

Preparation of activated diacetylenic acid-phospholipid liposomes. A solution of polydiacetylenic 2'-aminoethylamide and phospholipids (20-25 mg, in 10 mL of nitrogen-degassed chloroform, adjusted to 40, 50 or 60 mol% of diacetylene lipid) was rotary evaporated to dryness at 20 ^{O}C. Sodium phosphate (0.1 M, pH 7.2, containing 0.1 M sodium chloride, 40-50 mL, degassed with nitrogen) was added to give a total lipid concentration of 0.5 mg mL^{-1}, and the suspension gently shaken under nitrogen (150 rpm, 15 min) at 50 ^{O}C to suspend the lipid film. Representative lipid compositions used in the screening procedures are given in Table 1. The suspension was then sonicated (BioLogics Ultrasonic Homogenizer, 3.8 or 9.5 mm titanium probe, 35-40 W) in the dark under nitrogen at 40, 50 or 60 ^{O}C, for 20 min, centrifuged (14,000*g*, 15-20 min), filtered through a 0.2 or 0.25 μm nylon or polysulfone filter, then kept in the dark at 4 ^{O}C for 20-22 h. A solution of Sulfo-SMCC (0.1 mmol, in 1 mL water) was added over 10 min and the solution stirred (200 rpm) in the dark at 5 ^{O}C for 1 h, the solution concentrated by evaporation under vacuum then filtered through a 0.2 or 0.25 μm nylon or polysulfone filter to give a solution of maleimidyl-activated diacetylene-phospholipid liposomes (ca. 8-9 mg total lipids mL^{-1}).

Preparation of IgG-PDA-phospholipid liposomes. A solution of IgG (0.1, 0.2 or 0.3 mg in 1 mL of 0.1 M sodium phosphate buffer, pH 6, containing 50 mM sodium chloride and 5 mM EDTA) was incubated with 2-mercaptoethylamine (2 μL), with orbital shaking (200 rpm), at 37 ^{O}C for 1-2 h. The solution was cooled to 4 ^{O}C and the buffer exchanged (for 0.1 M sodium

phosphate, pH 7.2, containing 0.1 M sodium chloride and 5 mM EDTA) by repeated ultrafiltration (Whatman 2 mL polysulfone centrifugal ultrafilters, 10 K cutoff). The reduced IgG solution (total volume ca. 0.2 mL) was mixed with a solution of maleimidyl-activated diacetylene-phospholipid liposomes (ca. 3 mL, 8-9 mg mL^{-1}, freshly filtered through a 0.2 or 0.25 μm nylon or polysulfone filter) and the solution shaken (300 rpm) in the dark at 4 ^{O}C for 4-5 h. The liposome solution was vacuum degassed, then polymerized using a UV lamp (Pen-Ray, 254 nm, 4.1-6.8 J cm^{-2}) at 40-70 ^{O}C, 2-10 min, to yield a deep blue or blue-purple solution of polymerized IgG-PDA-phospholipid vesicles with an absorption maximum at 620-670 nm. SEM and dynamic light scattering analysis of the vesicles indicated diameters of 47-112 nm. The liposome solutions were carefully concentrated by evaporation under vacuum to ca. 38-42 mg mL^{-1} and stored under nitrogen in the dark at 4 ^{O}C until required.

Preparation of sol-gel precursors. Poly(glyceryl silicate) (PGS) of composition $SiO_{1.0}Glc_{1.2}$ was prepared according to the published procedure.[1] The corresponding glyceryl esters of DAPSOMe and BSPEOMe were synthesized as follows: DAPSOMe or BSPEOMe (20 mmol, in 50 mL of anhydrous methanol) was neutralized with glacial acetic acid (aliquots of mixture diluted 1:10 into water and pH measured), glycerol (0.5 mol per mol of methoxy groups) and concentrated hydrochloric acid (0.1 mL) added, and the mixture stirred (300 rpm) under reflux at 50 ^{O}C for 40-48 h. The solution was then rotary evaporated, first at 30 ^{O}C, then at 50 ^{O}C, to furnish the glyceryl ester as a viscous, pale yellow liquid which was completely miscible with water. GPPS was synthesized as follows: TPP (0.01 mol) was dissolved in nitrogen-degassed 3-glycidoxypropyltrimethoxysilane (3-GPTMOS, 0.2 mol) and the mixture heated under nitrogen to 80 ^{O}C. Glycerol (0.2 mol) was then added and stirring continued at 80 ^{O}C for 30 h, after which

time the temperature was lowered to 50 ^{O}C, concentrated hydrochloric acid (0.2 mL) added, and stirring continued for 20 h. The reaction mixture was then extracted with petroleum ether (4 x 50 mL), and the liquid siloxane rotary evaporated at 50 ^{O}C to give the required GPPS as a pale yellow gel.

Sol-gel encapsulation of IgG-PDA-phospholipid liposomes. In a typical procedure, finely powdered PGS (1 g) was mixed with water (0.3 g) and the gel left over ice for 10 min to form a viscous liquid. This was mixed with ice-cold solutions of DAPS-glyceryl ester (0-0.25 g, ca. 70% w/w solution in water), GPPS (0-0.5 g, 75% w/w solution in water), BSPE-glyceryl ester (0-0.5 g, 70% w/w solution in water), PVA (0-0.5 mL of 15% w/w solution in water, 70,000-100,000 molecular weight PVA), IgG-PDA-phospholipid vesicles (0.3-0.5 mL, ca. 38-42 mg mL^{-1}), and sodium phosphate (0.2 M, pH 7.5, 0.1 mL), and the mixture shaken vigorously (300 rpm). A control IgG-PDA-phospholipid-silica sol-gel mix was prepared using only PGS as the precursor. The sol-gel mixes were spotted as ca. 3-4 mm thick-film spots into the wells of 96-well polycarbonate microplates, or onto cellulose (Whatman, Grade 54), nylon (0.2 mm), polycarbonate (2 mm) or glass (1 mm) sheets using a manual spotter (5, 6 or 8 pins, ca. 100 μL pickup volume), or coated as ca. 1 mm thick, 0.5 x 1 cm rectangles onto cellulose or nylon, cellulose acetate or glass using a 1 mm silicone mask and a 1 cm diameter Teflon coating rod. The nylon sheet was washed with methanol, primed by incubation with a solution of 3-APTEOS and 3-UPTEOS (1% and 3% v/v respectively, in 2:1 methanol-water, 50 ^{O}C, 20 min), then washed with methanol and dried under nitrogen, prior to spotting. The polycarbonate sheet was washed with 1:1 methanol-ethanol, primed by incubation with a solution of 3-APTEOS and 3-UPTEOS (1% and 3% v/v respectively, in 2:1 methanol-water, 50 ^{O}C, 20 min), then washed with

methanol and dried under nitrogen, prior to spotting. The glass sheet was cleaned with aqueous ammonia-hydrogen peroxide (1 M ammonia and 0.5 M hydrogen peroxide, 60 ^{O}C, 1 h), then aqueous hydrochloric acid (1M, 70 ^{O}C, 20 min), washed with water, primed by incubation with a solution of 3-APTEOS and TMOS (1% and 4% v/v respectively, in 9:1 methanol-water, adjusted to pH 8 with acetic acid, 50 ^{O}C, 20 min), washed with methanol, then dried under nitrogen, prior to spotting. Compositions varying in the type and amount of diacetylene and phospholipid components, the content of IgG, the content of IgG-PDA liposomes, and the constitution of the sol-gel matrix were prepared as thick-film spot/rectangle arrays (typically 6 x 9, 15 x 9, and 12 x 7). The coated substrates were refrigerated at 4 ^{O}C until gelation (ca. 2-15 min), and aged in open or closed containers for 20-60 h prior to use. Aged, wet composites showed ca. 4-9% shrinkage and held ca. 35-40% w/w water, while aged, dry composites displayed ca. 10-17% shrinkage and retained ca. 9-14% w/w water.

Colorimetric immunosensing using sol-gel-IgG-PDA composites. The sensor arrays were washed with sodium phosphate buffer (50 mM, pH 7) at room temperature, gently blotted dry with filter paper, then exposed to protein solutions (0-2,000 pg mL^{-1}, prepared using 0.2 or 1.0 μg mL^{-1} stocks in 50 mM potassium phosphate, pH 7). The spectra (240-800 nm) and blue/red absorbances of the spots were measured (absorbance maximum of blue form at 620-670 nm, and that of red form at 510-560 nm) using a UV-VIS microplate reader (Bio-Tek, ELx808u) or UV-VIS spectrometer (Perkin Elmer, Lambda 800), and the colorimetric responses (CR) calculated using the equation CR = $(B_0 - B_1)/B_0$, where B = $A_{Blue}/(A_{Blue} + A_{Red})$ and $A_{Blue/Red}$ is the blue/red absorbance.[2] The fastest responses were generally obtained with sol-gel-PDA composites prepared using 2,4-TRCDA (CR of 0.9 within ca. 250-300 s), while sensors fabricated using 5,7-

DCDA and 5,7-TRCDA, 5,7-TCDA and PCDA were less responsive (CR of 0.9 within ca. 400-600 s, ca. 350-500 s, ca. 500-600 s and ca. 300-400 s, respectively).

References

[1] I. Gill, A. Ballesteros. *J. Am. Chem. Soc.* **1998**, *120*, 8587-8598.

[2] W. Spevak, J. O. Nagy, D. H. Charych. *Adv. Mater.* **1995**, *7*, 85-89.

Table 1. Representative liposome compositions (mol% lipid) used in studies

Diacetylenic 2'ethylamide	DMPG	DMPC	DPPC	DMPE	DPPE	OPPE	*LPPE*
70	10 or 20	20 or 10					
60	10, 20 or 30	30, 20 or 10					
50	20 or 30	30 or 20					
40	20 or 40	40 or 20					
30	30 or 40	40 or 30					
70	10 or 20		20 or 10				
60	10, 20 or 30		30, 20 or 10				
50	20 or 30		30 or 20				
40	20 or 40		40 or 20				
30	30 or 40		40 or 30				
70		10 or 20	20 or 10				
60		10, 20 or 30	30, 20 or 10				
50		20 or 30	30 or 20				
40		20 or 40	40 or 20				
30		30 or 40	40 or 30				
70		10 or 20		20 or 10			
60		10, 20 or 30		30, 20 or 10			
50		20 or 30		30 or 20			
40		20 or 40		40 or 20			
30		30 or 40		40 or 30			
70		10 or 20			20 or 10		
60		10, 20 or 30			30, 20 or 10		
50		20 or 30			30 or 20		
40		20 or 40			40 or 20		
30		30 or 40			40 or 30		
70		10 or 20				20 or 10	
60		10, 20 or 30				30, 20 or 10	
50		20 or 30				30 or 20	
40		20 or 40				40 or 20	
30		30 or 40				40 or 30	
70		10 or 20					*10 or 20*
60		10, 20 or 30					*10, 20 or 30*
50		20 or 30					*20 or 30*
40		20 or 40					*20 or 40*
30		30 or 40					*30 or 40*
70			10 or 20	20 or 10			
60			10, 20 or 30	30, 20 or 10			
50			20 or 30	30 or 20			
40			20 or 40	40 or 20			
30			30 or 40	40 or 30			
70			10 or 20		20 or 10		
60			10, 20 or 30		30, 20 or 10		
50			20 or 30		30 or 20		
40			20 or 40		40 or 20		
30			30 or 40		40 or 30		