mono-thiophene (*N*-[(6-(thien-3-yl)hexanoyloxy]-pyrrolidine-2,5-dione; see Scheme 1) instead of the bithiophene derivative, we have been able to prepare analogous glucose oxidase-modified polymer films. The functionalized polythiophene film has been obtained using a similar multisweep regime, however, with potential scans up to a vertex potential of 1.7 V vs. SCE, reflecting the higher potential of the radical cations formation. The second step, the covalent immobilization of the enzyme is of course equivalent and independent from the specific needs for the formation of the polymer film. The obtained enzyme electrodes show a slightly lower response as those obtained with the functionalized poly(bithiophene) films, despite there are double active ester groups available to react with the enzyme. Probably, due to the size of an individual enzyme molecule the increased number of binding sites at the electrode surface does not concomitantly increase the immobilized enzyme activity.

To conclude, we have shown that, despite the high oxidation potential of thiophene and its derivatives, polythiophene films can be advantageously used for the entrapment or covalent immobilization of enzymes. This was realized by entrapment of glucose oxidase using dimeric or trimeric thiophenes as parent compounds for the polymerization process in CH_3CN/water mixtures or, according to a two-step procedure, based on functionalized polythiophene or poly(bithiophene) films and covalently bound enzyme. In principle, polythiophene as immobilization matrix in amperometric enzyme electrodes has at least two advantages over other conducting polymer films: First, derivatization of thiophene in the 3-position and the synthesis of oligomers is much easier than for pyrrole. Second, due to the higher redox potential of the polymer film itself, deterioration e.g. by enzymatically generated H_2O_2 is less probable. This is even more important for a next sensor generation, in which the conducting polymer is expected to act as molecular wire between the active site of the enzyme and the electrode surface. Possibly, the improved stability of polythiophene against oxidation in ambient environments will allow the construction of biosensors with improved sensor characteristics and life-time.

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Stabilization of Amorphous Calcium Carbonate by Specialized Macromolecules in Biological and Synthetic Precipitates**

By *Joanna Aizenberg,** *Gretchen Lambert*, *Lia Addadi,** and *Stephen Weiner**

Some organisms form stable minerals that would never precipitate under ambient conditions in an inorganic environment, or, if formed, are unstable precursors of more stable compounds.[1,2] One of the most fascinating examples is biogenic amorphous calcium carbonate, because its transformation into crystalline polymorphs is not only thermodynamically favored, but also kinetically fast.[3]

We report here an example of a single skeletal element, spicules from the calcareous sponge *Clathrina*, composed of crystalline calcite in one layer and stable amorphous $CaCO_3$ in another. Differential dissolution of the amorphous phase of these spicules released macromolecules with proteins rich in glutamic acid (and/or glutamine), serine, glycine and polysaccharides. Similar macromolecules are present in spicules of the ascidian *Pyura pachydermatina* which are composed entirely of amorphous $CaCO_3$. We show that the presence of small amounts of these macromolecules in saturated solutions of $CaCO_3$ results in the formation in

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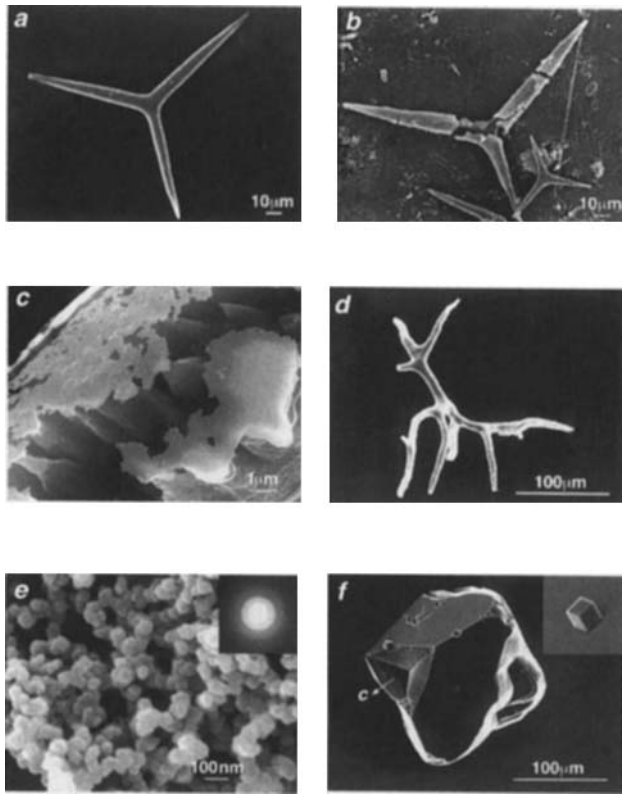

Fig. 1. Scanning electron micrographs of: a) Intact triradiate spicule from the calcereous sponge *Clathrine sp.* b) Heated *Clathrina* spicule, showing two different $CaCO_3$ phases; an outer amorphous layer and a stable calcitic core. Note the separated calciic core indicated by an arrow. c) Partially KOH etched *Clathrina* spicule reveals a thin, apparently crystalline, outer sheath. d) Antler spicule from the ascidian *Pyura pachydermatina*. The entire spicule is composed of amorphous $CACO_3$. e) Synthetic amorphous calcium carbonate precipitated in the presence of 2.0 μg/ml proteins extracted from the amorphous phase of *Clathrina* spicules. Insert: electron diffraction pattern of the precipitate embedded in vitrified ice, showing its amorphouse character. f) Synthetic calcite crystal grown in the presence of 2.0 μg/ml proteins extracted from the calcitic core of the *Clathrina* spicules. Note that the acidic proteins intefere with calcite growth in the *c* axis direction, causing the development of the new {001} face. Insert: Synthetic calcite crystal of regular morphology grown in the absence of additives.

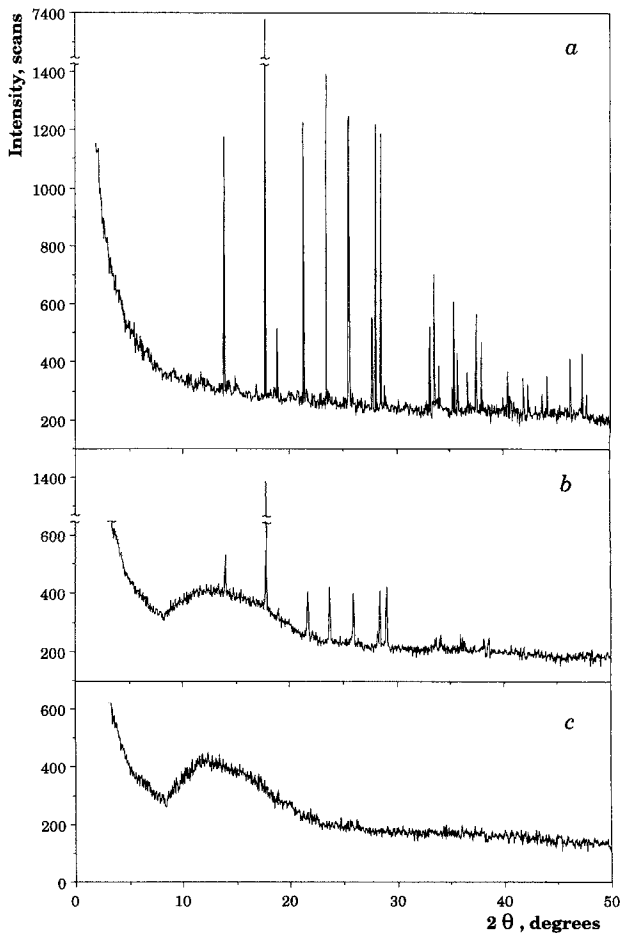

Fig. 2. Synchrotron powder X-ray diffraction analyses of different $CaCO_3$ phases. a) Geological calcite. b) *Clathrina* spicules. Note the presence of the sharp calcite reflections and the base-line rise due to the amorphous material. From the comparison of the intensities of the different crystallographic peaks with those of an equal volume of pure calcite, the amount of crystalline material in the *Clathrina* spicules was found to be about 20%. c) *P. pachydermatina* spicules.

vitro of stable amorphous $CaCO_3$. In contrast, the proteins extracted from the crystalline calcitic phase are rich in aspartic acid (and/or asparagine) and induce calcite formation.

The formation of composite structures in which amorphous and crystalline phases coexist apparently endows the biogenic material with advantageous properties, that may be interesting to investigate. The ability to control the formation of stable amorphous phases may also have application to different synthetic materials.

The calcareous sponges (Porifera) form a wide variety of spicules, each of which is generally thought to be composed of a single crystal of Mg-bearing calcite.[4–8] Significant differences have, however, been observed between the spicule mineral and inorganically formed calcite crystals. Most of the sponge spicules have conchoidal rather than smooth cleavage surfaces[5,7] and lower specific gravities.[5–7] Some tend to disintegrate when heated, presumably due to loss of water.[5,7] However, polarized light microscopy,[5] etching,[4,8] overgrowth[4,7,9] and X-ray diffraction studies[6] all support the notion that the spicules are composed of calcite. The sponge *Clathrina* forms regular triradiate spicules (Fig. 1a) that were observed to form from non-birefringent material deposited in three separate vesicles, which upon fusion becomes completely crystalline.[10]

Our attention was drawn to the fact that an additional amorphous mineral phase may be present, by the observation that the intensities of the diffracted beam from single *Clathrina* spicules accounted for only about 20% of the expected value of an equivalent volume of a pure single calcite crystal.[11] A high-resolution powder X-ray diffraction pattern of *Clathrina* spicules using synchrotron radiation revealed, in addition to the sharp calcite reflections (Fig. 2a), an intense but broad peak around $2\theta = 15°$ (Fig. 2b) indicative of the presence of an amorphous phase. An infrared spectrum of the intact spicules (Fig. 3c)

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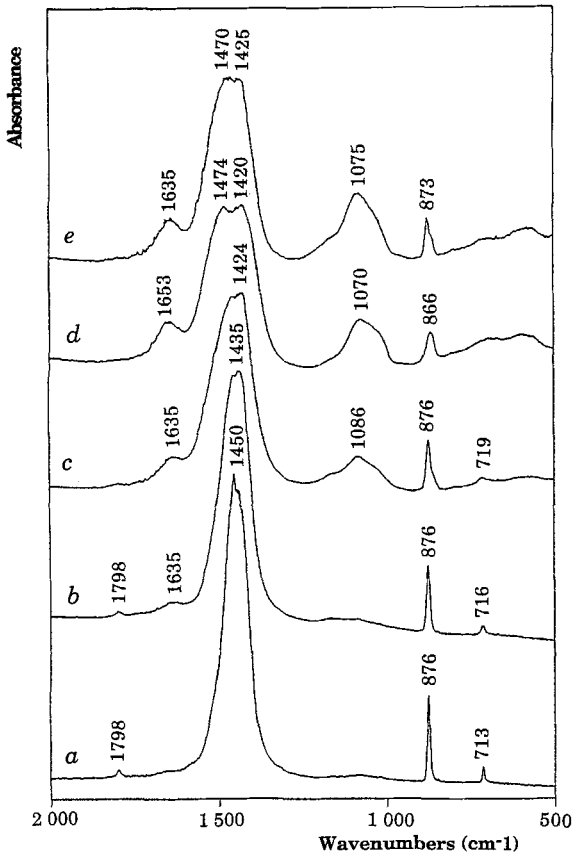

Fig. 3. Infrared spectra of calcium carbonates of different origin. a) Geological calcite. b) Separated calcitic cores from the *Clathrina* spicules. c) Intact *Clathrina* spicules. Note the atypical broadening of the calcitic peaks and the additional absorption at 1080 cm^{-1}. d) *P. pachydermatina* spicules. e) Synthetic amorphous $CaCO_3$ grown in the presence of the macromolecules extracted from the amorphous layer of *Clathrina* spicules.

shows a broadening of all the characteristic calcitic peaks (Fig. 3a), as well as additional broad peaks around 3430 and 1080 cm^{-1}, typical of hydrated amorphous calcium carbonate.[12]

Upon heating ($\sim 100\,^{\circ}C$) in a drop of water, the spicules decompose into two separate domains, with a smooth stable inner core (~ 8–15 vol.-%) and a heavily corroded outer layer (Fig. 1b). Under mild acidic conditions (pH ~ 5) well defined triangular etch figures indicative of crystalline calcite are formed on the surface of the central part of the spicules, in contrast to the irregular shapeless etch patterns of the ray extremities. The outer layer can be solubilized in dilute (< 5 mM) $CaCl_2$ solution or doubly distilled water, while the core remains intact.

The infrared spectrum of the separated core material contains only calcitic absorption bands (Fig. 3b). One puzzling observation is that freshly extracted un-etched spicules show a clear birefringence.[5] This implies that the outer layer must possess at least a thin exterior crystalline sheath. KOH etching (pH ~ 9), which does not affect pure calcite,[5] does reveal a thin outer sheath (Fig. 1c). On the basis of the above observations, we deduce that the spicule is composed of a core of calcite embedded in a thick amorphous calcium carbonate layer and is apparently covered by a thin calcitic sheath. We can infer from Minchin's observations[10] that the initial mineral deposited is amorphous and stable, and that the deposition of the crystalline core material and of the external sheath occurs in separate domains as a later event.

Specialized macromolecules are found within the calcite crystals of many organisms.[13,14] Their presence is known to affect the mechanical properties of calcite,[15] and in the case of calcareous sponge spicules to be involved in shaping their morphology during growth.[9,11] The protein content of *Clathrina* spicules is 0.12 wt.-%. In order to study their possible effects on the observed phase stabilization, the macromolecules associated with the two different mineral phases were extracted by differential dissolution. The amino acid compositions of the two protein assemblies are quite different (Table 1). The amorphous material contains GlX-, Ser- and Gly-rich proteins, whereas those in the crystalline core are AsX-rich. The latter composition is typical of the proteins associated with many other calcitic and aragonitic mineralized tissues.[16] Infrared spectra of the two macromolecular fractions show that the amorphous phase macromolecules are also rich in polysaccharides, while the main constituent of the core fraction is not.

To further investigate the ability of organisms to stabilize a metastable amorphous phase, we studied the ascidian *Pyura pachydermatina* (Chordata), which forms antler-shaped and dog bone-shaped spicules. The antler-shaped spicules (Fig. 1d) were found to be composed entirely of amorphous $CaCO_3$ (Fig. 2c, 3d). Spicules composed of

Table 1. Amino acid compositions of the proteins extracted from ascidian antler spicules and triradiate sponge spicules.

Amino acids	*Pyura pachydermatina* antler spicules	*Clathrina* triradiate spicules	
		Amorphous layer	Crystalline core
Asx*	**7.8**	**7.2**	**28.5**
Thr	4.6	4.3	3.5
Ser	**18.0**	**19.2**	**8.8**
Glx**	**16.8**	**17.7**	**14.3**
Pro	3.2	5.4	4.2
Gly	**19.6**	**19.9**	**10.8**
Ala	8.0	8.4	10.5
Cys	0.1	-	0.2
Val	3.5	3.5	3.5
Met	0.6	-	2.1
Ile	2.4	1.7	2.0
Leu	3.5	2.3	2.9
Tyr	1.8	0.8	1.6
Phe	1.7	1.0	2.3
Lys	3.0	4.1	1.7
His	3.1	3.2	1.1
Arg	2.3	1.1	2.0

*Asx - combination of Asp and Asn.

**Glx - combination of Glu and Gln.

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amorphous $CaCO_3$, as well as of a variety of other minerals, are known to be formed by the *Pyuridae*.[17] Amino acid and infrared analyses of the macromolecules within the antler spicules (0.09 wt.-% protein) showed that they are also polysaccharide-rich proteins with amino acid compositions that are remarkably similar to those from the amorphous layer of the *Clathrina* sponge spicules (Table 1).

We added small amounts of the macromolecules from within the *Clathrina* spicule amorphous phase to solutions saturated with respect to $CaCO_3$. This resulted in complete inhibition of crystallization at protein concentrations of 1.0 μg/ml. A similar phenomenon occurred with the macromolecules extracted from the *Pyura* spicules, but at protein concentrations of 0.5 μg/ml. A few irregular crystals were formed when the solutions were doped with lower concentrations of proteins. Precipitation of a non-birefringent $CaCO_3$ phase occurred with further increases in protein and calcium concentrations (2 μg/ml and 25 mM, respectively). The yield of synthetic amorphous $CaCO_3$ in this experimental setup using *Clathrina* proteins was estimated by atomic absorption (i) from the difference in the calcium contents between the solutions before and after precipitation. These were found to be 1000 and 690 μg/ml Ca, respectively. (ii) Directly from the yield of dissolved precipitate which was 300 μg/ml Ca. The yield of precipitate is thus 30.0–31.0 wt.-%. This corresponds to the formation of 775 μg/ml of $CaCO_3$. The maximum possible fraction of protein in the final solid is 0.26 wt.-%, assuming all 2 μg/ml protein is incorporated. We therefore conclude that the precipitate is composed of > 99.5 % $CaCO_3$ and water.

Figure 1e shows the spherical particles formed in the presence of 2.0 μg/ml proteins from the amorphous layer of *Clathrina* spicules. A material of the same appearance was obtained with the addition of 1.0 μg/ml soluble proteins from the *Pyura* antler spicules. The size of the spherules is about 50–70 nm. The amorphous character of this material was unequivocally confirmed by infrared spectroscopy (Fig. 3e), as well as by the absence of a discernible electron diffraction pattern from the precipitate embedded in vitrified ice (Fig. 1e). No transformation of the air-dried synthetic amorphous $CaCO_3$ into the favored crystalline forms was observed during three months. If the precipitate is kept in the mother liquor, some calcite crystals are formed in addition to the amorphous material after three to four days. In contrast, under the same experimental conditions numerous calcite crystals with well-developed (001) crystallographic faces were obtained in the presence of 0.5–4.0 μg/ml of the AsX-rich macromolecules from the calcitic cores of the *Clathrina* spicules (Fig. 1f). In the latter case, macromolecules do not inhibit crystallization, but modulate crystal morphology by interfering with calcite growth in the direction of the *c*-axis.[11,13]

These results suggest that the specialized sugar-, GlX-, Gly- and Ser-rich macromolecules associated with the amorphous calcium carbonate phases in the ascidians and the sponges are responsible for inhibition of crystallization, the precipitation of an amorphous metastable phase, and its stabilization. Following the observation that low concentrations of these proteins in solution drastically reduce the amount of calcite crystals formed, we suggest that the macromolecules poison the growth of embryonic nuclei of crystallization. Suppression of calcite nucleation thus presumably allows the concentration of the ions in solution to increase to the point where the amorphous phase becomes supersaturated. This mechanism is analogous to that proposed for the stabilization of amorphous calcium phosphate by $Mg^{2\oplus}$ and ATP.[18] In vitro precipitation of almost exclusively amorphous calcium carbonate with higher concentrations of protein and in the absence of any other additives indicates that it is sufficient to add only macromolecules from biogenic amorphous phases to maintain metastable amorphous $CaCO_3$ in a stable state. The manner in which the macromolecules stabilize the amorphous phase is still to be understood.

Stable biologically produced amorphous $CaCO_3$ is known to be formed by members of the plant kingdom, Cyanobacteria, Mollusca, Arthropoda and Chordata.[1,19,20] The difficulties involved in detecting amorphous $CaCO_3$ when it is associated with one or more of the crystalline polymorphs in the same skeletal element probably account for the fact that this phase remained undetected in *Clathrina* spicules for more than 100 years. We therefore suspect that biologically formed amorphous calcium carbonate may well be a lot more widespread than commonly supposed. It is thought to function as temporary storage sites in some organisms, as a precursor of crystalline $CaCO_3$ polymorphs in others, and also as a functional material in skeletal elements where it is present as a stable entity.[1,2,19]

Amorphous materials have the advantage of being isotropic and less brittle. On the other hand, their crystalline counterparts are harder and less soluble. The combination of the two phases, as in *Clathrina* spicules, may offer some advantages that we do not yet fully appreciate. If the mechanisms by which the different glycoproteins control the formation and stabilization of the different phases could be elucidated, this knowledge may well have practical applications in the field of materials science.

Experimental

Materials: Individual *Clathrina* spicules (Atlit, Israel) were isolated and cleaned as described previously [11]. Spicules suspended in a drop of doubly distilled water were placed on an aluminum SEM stub and heated until all the water evaporated. The volume fraction of the remaining crystalline parts in the entire spicules was measured from the micrographs. The evaluated crystalline fraction is 11 ± 5 %. Antler spicules from *Pyura* (Otago Harbour, Portobello, New Zealand) branchial sac tissue were isolated as described previously [21], except that fresh unfixed tissue was used. The samples were examined in a JEOL 6400 SEM after gold coating.

Extraction procedure: Cleaned *Clathrina* spicules were placed on a rocking table for 3 days in doubly distilled water. The undissolved calcitic cores were separated by low speed centrifugation. Extensive dialysis of the solution (Spectrapor 3 dialysis tubing), followed by lyophilization, yielded the macromolecules from the amorphous phase. The calcitic cores were dissolved separately as described previously[11] to yield an additional protein fraction. Macromolecules from within the *Pyura* antler spicules were extracted and analyzed as described for the sponge spicule amorphous phase. Amino acid

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compositions were determined using ninhydrin detection (Dionex BIOLC) after hydrolysis in 6 N HCl.

Precipitation of $CaCO_3$: In vitro $CaCO_3$ precipitation was performed by slow diffusion of ammonium carbonate vapor in a 25 mM calcium chloride solution doped with aliquots of the proteins extracted from the biogenic amorphous $CaCO_3$ phases and the calcitic cores of sponge spicules. Protein concentrations were determined from amino acid composition analyses.

Atomic absorption measurements: After precipitation, the mother liquor (0.75 ml) was quantitatively separated and diluted to 25 ml. The precipitate was washed in the experimental dish, dried and dissolved in 1 N HNO_3. The solution obtained was then diluted to the same volume as the mother liquor. The amounts of calcium were measured in the initial solution, in the solution that underwent precipitation and in the dissolved precipitate, using atomic absorption (Perkin Elmer 5100 GFAAS).

X-ray measurements: The powder diffraction data were collected at the National Synchrotron Light Source in Brookhaven National Laboratory, on beam line X7B (operated under contract DE-AC02-76CH00016 with the US Department of Energy). The samples were finely ground, and 0.3 mm glass capillaries were filled completely with the powder to ensure that the same amount of material was subjected to the X-ray beam in each experiment. Narrow slit measurements were performed with a step size of 0.01° and sampling time of 10 s/step using a Huber diffractometer. The data presented are after subtraction of the corresponding reference measurements of the empty capillaries.

Infrared analyses: Ground samples were dispersed in 7 mm KBr pellets. The infrared spectra were collected using a computer-controlled spectrometer (MIDAC Corporation, Costa Mesa, CA, USA). The data presented are after subtraction of the corresponding reference KBr spectra.

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Donor–Acceptor Substituted Tetraethynylethenes**

By *Rik R. Tykwinski, Martin Schreiber, Volker Gramlich, Paul Seiler,* and *François Diederich**

Derivatives of tetraethynylethene (TEE, 3,4-diethynyl-hex-3-ene-1,5-diyne)[1] constitute a versatile "molecular construction kit" which provide access to conjugated acetylenic acyclic[2] and macrocyclic[3] molecular scaffolds with unusual electronic and structural properties as well as linearly-conjugated polymers with the polytriacetylene (PTA) backbone.[4]

Tetrakis(phenylethynyl)ethene[5] has been shown to form highly ordered charge transfer complexes with π-acceptors in the solid state and in solution.[6] Substituents in all TEE derivatives reported to date were either silyl, alkyl, alkynyl, phenyl, and alkylphenyl groups, or halogens. To enhance the interest in TEEs for potential applications as conjugated materials for electronics and photonics,[7] we became interested in preparing derivatives with electron donor and/or acceptor groups. By varying the attachment of *p*-donor and *p*-acceptor substituted phenyl rings to the planar TEE chromophore, different donor–acceptor conjugation paths are generated (Fig. 1). *Trans*- and *cis*-orientations of the substituents provide one-dimensional linear donor–acceptor conjugation paths (**1** and **2**), geminal substitution affords one-dimensional cross-conjugation (**3**), and substitution at all four terminal alkynes gives full, two-dimen-

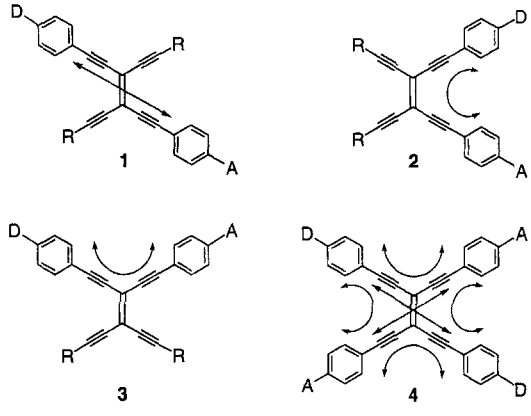

Fig. 1. Schematic representations of possible conjugation paths in donor–acceptor substituted tetraethynylethenes (TEEs). D = donor. A = acceptor. R = $Si(i\text{-}Pr)_3$ or $SiMe_3$.

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