Substrate-Initiated Synthesis of Cell-Penetrating Poly(disulfide)s

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Abstract

Lessons from surface-initiated polymerization are applied to grow cell-penetrating poly(disulfide)s directly on substrates of free choice. Reductive depolymerization after cellular uptake should then release the native substrates and minimize toxicity. In the presence of thiolated substrates, propagators containing a strained disulfide from asparagusic or, preferably, lipoic acid and a guanidinium cation polymerize into poly(disulfide)s in less than 5 min at room temperature at pH 7. Substrate-initiated polymerization of cationic poly(disulfide)s and their depolymerization with dithiothreitol causes the appearance and disappearance of transport activity in fluorogenic vesicles. The same process is further characterized by gel-permeation chromatography and fluorescence resonance energy transfer.

Cell-penetrating peptides (CPPs) are short, polycationic peptides or protein domains that are used by viruses to enter cells.[1,2] Their unique ability to transport linked substrates across lipid bilayer membranes has attracted great interest in biomedical applications. Substrates of varying sizes and properties, e.g., small fluorophores to proteins and quantum dots, have been successfully transported into cells using CPPs. The mechanism of cellular uptake is under debate, currently favored are endocytosxis (i.e., macropinocytosis) or passive diffusion across the membrane, depending on conditions. Multiple, moderately hydrophobic cations seem to be all that is needed. Guanidinium cations, as in arginine, are most common, alternatives include ammonium or phosphonium cations.[1] The originally peptidic oligomer backbone has been extensively varied, covering oligocarbamates, β-peptides and several variations of synthetic polymers.[1] Currently, cell-penetrating poly(disulfide)s are emerging as the cell-penetrating molecules of the future because their cytosolic degradation liberates the substrate and eliminates toxicity, one of the key disadvantages associated with CPPs.[3-5] However, cell-penetrating poly-(disulfide)s have so far been used mainly in noncovalent polyplexes for gene transfection, and covalent attachment of substrates would be difficult with their preparation methods. We have found recently that poly(disulfide)s can be grown directly on solid substrates by surface-initiated ring-opening disulfide-exchange polymerization.[6] Therefore, we wondered whether the same methodology could be used to

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prepare cell-penetrating poly(disulfide)s with covalently attached substrates in solution (Figure 1). Probes or drugs that contain thiol group but cannot penetrate cells without assistance are the ideal substrates, which could serve as an initiator to be appended with a membrane-active poly(disulfide). Thiolated siRNA, for instance, is commercially available. The generality of this approach promises a conceptually innovative solution for a central current challenge, i.e., the noninvasive, nontoxic delivery of unmodified substrates in well-defined, covalent systems rather than complex, noncovalent formulations. In this initial report on the topic, we describe the design, synthesis and evaluation of propagators for the substrate-initiated synthesis of cell-penetrating poly(disulfide)s. Their formation in less than 5 min at pH 7 and their depolymerization with 10 mM dithiothreitol (DTT) can be followed directly as appearance and disappearance of transport activity in fluorogenic vesicles.

To ultimately combine surface-initiated polymerization[6] with cellular uptake,[1-5] we prepared the strained disulfides 1–4 as possible propagators, thiols 5–7 as initiators, and the iodoacetamides 8 and 9 as terminators (Figure 1). The synthesis of all new compounds was straightforward and is described in detail in the Supporting Information (SI Schemes S1–S7, Figures S10–S23).[7] Only freshly prepared material was used.

Fluorogenic vesicles are convenient analytical tools to follow reactions with minimal effort and maximal speed.[8] Here, EYPC-LUVs⊃CF, i.e., large unilamellar vesicles (LUVs) composed of egg yolk phosphatidylcholine (EYPC) and loaded with 5(6)-carboxyfluorescein (CF), were used. EYPC-LUVs⊃CF report CF release as fluorescence recovery because local dilution reduces self-quenching.

The addition of propagator 1 to EYPC-LUVs⊃CF caused CF release only above a relatively high EC_{50} = 129.9 ± 0.7 μM (Figures 2A, B, ○, S1–S3, S6). Ring-opening disulfide exchange polymerization[3] of 1 (100 mM, pH 7, 1 M triethanolamine (TEOA) buffer), initiated with thiol 5 (5 mM), was followed by adding aliquots of the reaction mixture to EYPC-LUVs⊃CF after termination with iodoacetamide 9. Rapid fluorescence recovery was observed with increasing reaction time (Figure 3A, ●). At saturation, dose response curves were recorded for the obtained polymers (Figures 2A, B, ●, S1–S4). An EC_{50} = 3.2 ± 1.6 μM calculated to a 40-fold increase in activity upon substrate-initiated polymerization of propagator 1 with initiator 5 and terminator 9 (Figures 2B, ●, S2–S4).

According to activity in EYPC-LUVs⊃CF, substrate-initiated polymerization of propagator 1 was accomplished in less than 5 min (Figure 3A, ●). Polymerization was better in the presence than in the absence of initiator 5 (Figures 3A, S2–S4). This conclusion was supported by gel-permeation chromatography (GPC). Polymers obtained from propagator 1 in the presence of initiator 5 were of high molecular weight (M_w = 62.7 kDa) and dispersity (PDI = 1.83, Figure 3B, solid). Considering increasing transport activity with polymer length but less predictable length-dependence of cellular uptake of CPPs, high molecular weight and dispersity compared to commercially available polyarginine (M_w = 16.7 kDa, PDI = 1.7, Figure 3C, solid) were both very desirable characteristics. The same was true for the disappearance of all activity in EYPC-LUVs⊃CF within minutes of incubation with 10 mM DTT (Figure S7).[7] This is in the range of cytosolic glutathione (~5 mM) and thus confirms the previously reported biodegradability of cell-penetrating poly(disulfide)s.[3,4]

Without initiator, weaker absorbance, i.e., lower yield of polymer, at lower M_w ~ 16.2 kDa was observed (PDI = 2.1, Figure 3B, dashed).

The polymerization of propagator 1 was analyzed systematically from pH 5 to pH 9 and concentrations from 25 to 200 mM in the presence and the absence of 5 mM initiator 5 (pK_a ~ 9.5, Figure 4A, B). Best results were obtained with 100 mM 1 in 1 M TEOA, pH 7 (Figure 4A, B, dotted lines). At lower pH and concentrations, the substrate-initiate polymerization was incomplete, at higher pH, random polymerization without initiator as well as precipitation started to interfere.

The bell-shaped dependence on initiator concentration was in agreement with the formation of fewer polymers at low and more but shorter and thus less active ones at high initiator concentrations (Figure 4C). Corroborative evidence for the incorporation of the initiators into the polymers was obtained by GPC. Polymers obtained from propagator 2 and increasing concentrations of initiator 5 gave polymers with decreasing molecular weight and dispersity (Figure 4D). Moreover, polymers obtained with Cys-Trp initiator 6 showed the tryptophan emission in the GPC peak. The relative Trp emission increased with decreasing molecular weight, that is decreasing polymer/initiator ratio (Figure S8).[7]

The substrate-initiated polymerization of propagators 1 and 2 with the strained disulfides from lipoic acid was straightforward to control and optimize. The disulfides from asparagusic acid are ideal for surface-initiated polymerization[6] but turned out to be too reactive[9] for substrate-initiated polymerization in solution. Independent of their backbone, propagators 3 and 4 more easily polymerized with less difference between substrate-initiated and random polymerization without initiator (Figure S6). Moreover, cell-penetrating poly(disulfide)s obtained from lipoyl propagators 1 and 2 were active in EYPC LUVs, whereas the less lipophilic polymers from asparagusyl propagators 3 and 4 were poorly active. However, like arginine-rich CPPs,[2] all polymers could be activated in EYPC LUVs by counterions such as pyrenebutyrate (Figures S5, S6).[7]

To probe for substrate-initiated polymerization also with the less perfect asparagusyl propagators, fluorescence resonance energy transfer (FRET) was considered as a method complementary to the functional studies with fluorogenic LUVs and GPC described above for the preferable lipoyl propagators. Polymerization of propagator 4 in CHCl$_3$/DMF 3:1 was initiated with the yellow, green-fluorescent naphthalenediimide (NDI)[6] fluorophore 7 (λ_{ex} = 469 nm, λ_{em} = 484 nm) and 0.25% Hünig base (DIEA) as base, and terminated with the red NDI 8 (λ_{ex} = 552 nm, λ_{em} = 582 nm, Figure 5). With increasing polymerization time, the FRET emission at λ_{em} = 582 nm in CHCl$_3$ decreased (Figure 5). This decrease was consistent with increasing distance between initiator and terminator with increasing reaction time. Depolymerization with DTT caused nearly complete disappearance of FRET (Figure 5, dotted). These results further demonstrated the incorporation of the terminator in the polymer.

In summary, substrate-initiated polymerization of cell-penetrating poly(disulfide)s is introduced as a conceptually new approach to cellular uptake. Two types of propagators and four unrelated methods to prove direct growth of polymers on substrates are described.

Namely, (a) polymers obtained with and without initiators are different, (b) the dependence on initiator concentration is bell-shaped, (c) labeled initiators are eluted with polymers in GPC, and (d) FRET between donating initiators and accepting terminators decreases with polymerization time. Ring-opening disulfide exchange polymerization with propagators derived from lipoic acid is facile to control (pH, concentration of initiators, propagators, etc.) and gives polymers with high, stimuli-responsive transport activity in neutral lipid bilayers, whereas propagators derived from asparagusic acid are too reactive and give polymers that require counterion activation for function. With these complete, clear and consistent results, the newly introduced system is ready for cellular uptake experiments[10] and copolymerization studies[1a,6b,f] to modulate the properties of the cell-penetrating poly(disulfide)s.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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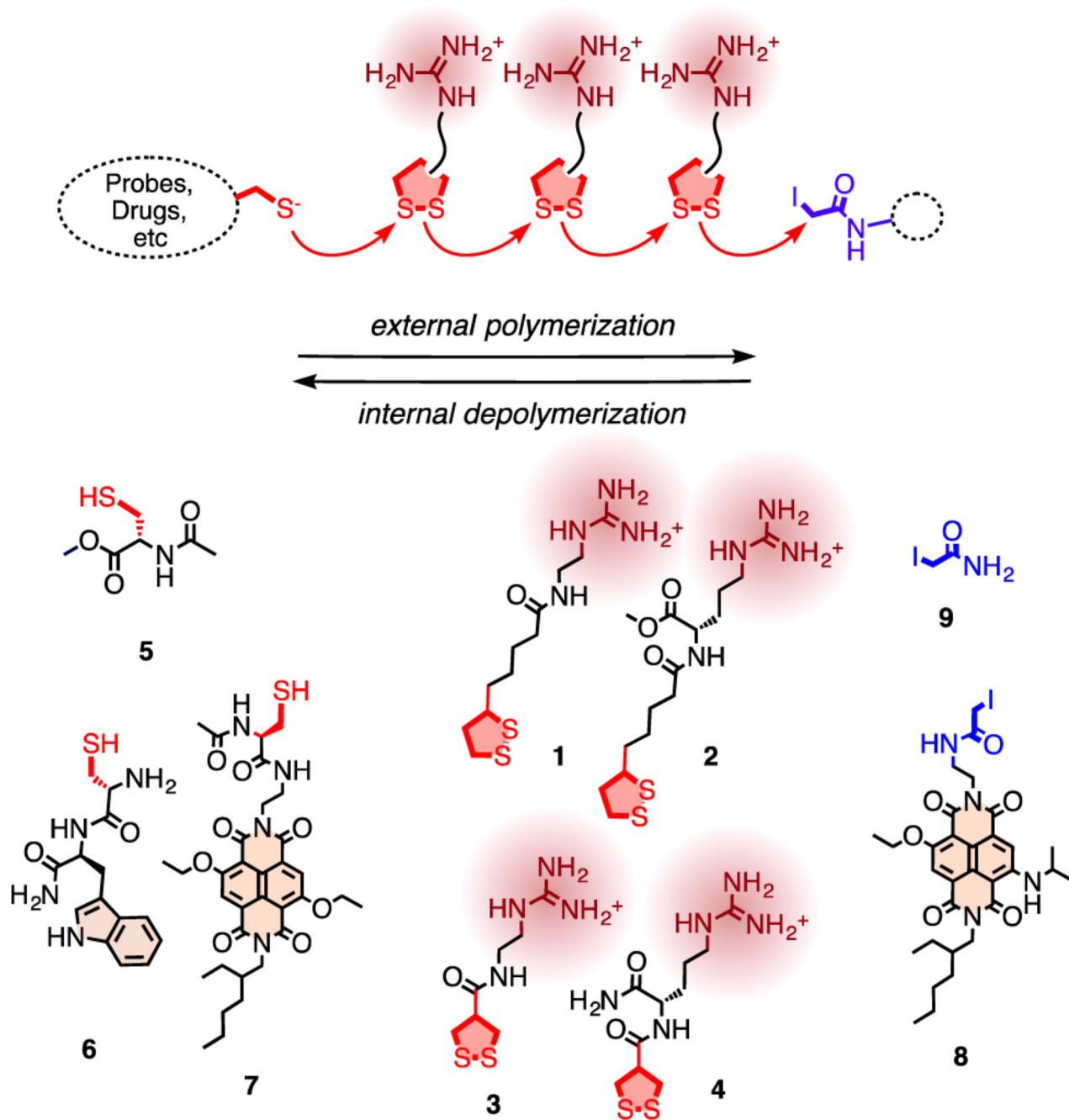
Figure 1.
For the covalent delivery of unmodified probes, guanidinium-containing propagators (e.g., 1–4) are polymerized on thiolated substrates (e.g., 5–7) and terminated with iodoacetamides (e.g., 8, 9). After uptake, cell-penetrating poly(disulfide)s are degraded by reductive depolymerization to eliminate toxicity and release the unmodified substrate.
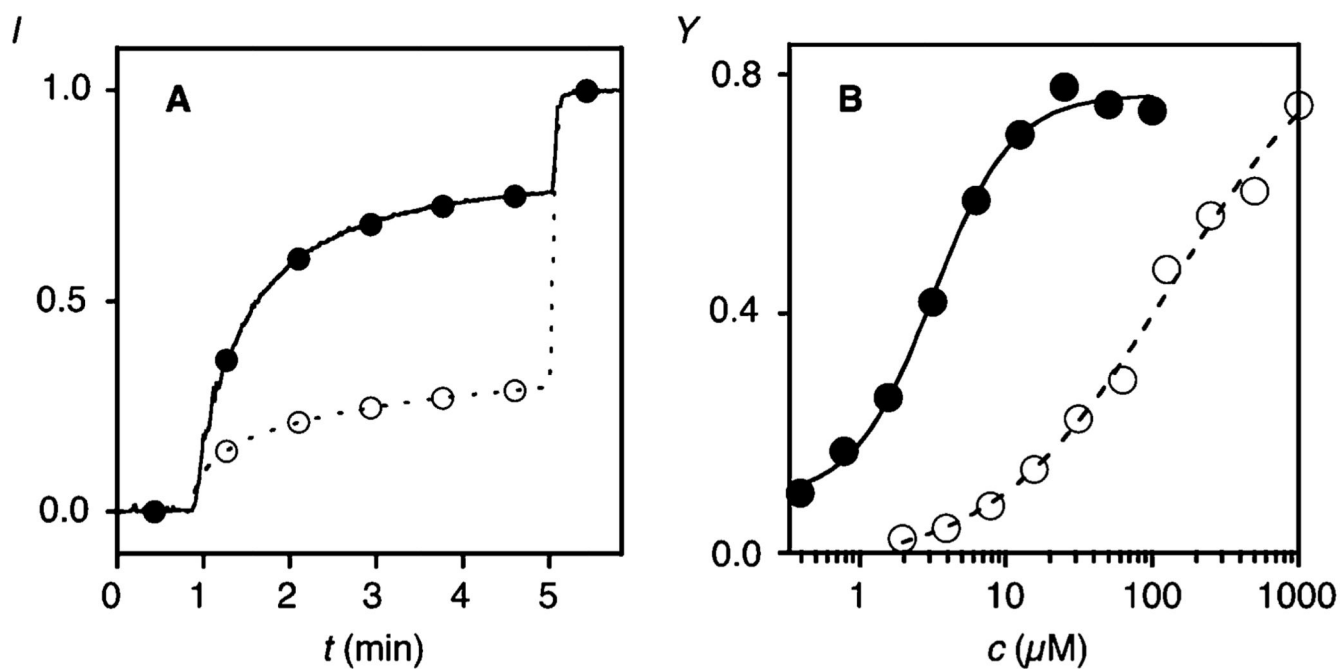
Figure 2.
Activity of monomers (○) and polymers (●). (A) Change in CF emission intensity *I* during the addition of reaction mixtures with and without initiator 5 (50 s, 75 μM final guanidinium (monomer 1) concentration) and excess Triton X100 (300 s) to EYPC-LUVs⊃CF. Reaction mixtures: 100 mM 1, 0 (○) or 5 mM 5 (●), 1 M TEOA, pH 7, 10 min; termination: 5 mM 9. (B) Transport activity *Y* of 1 before (○) and after polymerization (●, 5 mM 5) with increasing concentration of guanidinium cations (*Y* = *I* before lysis in A (~5 min)).
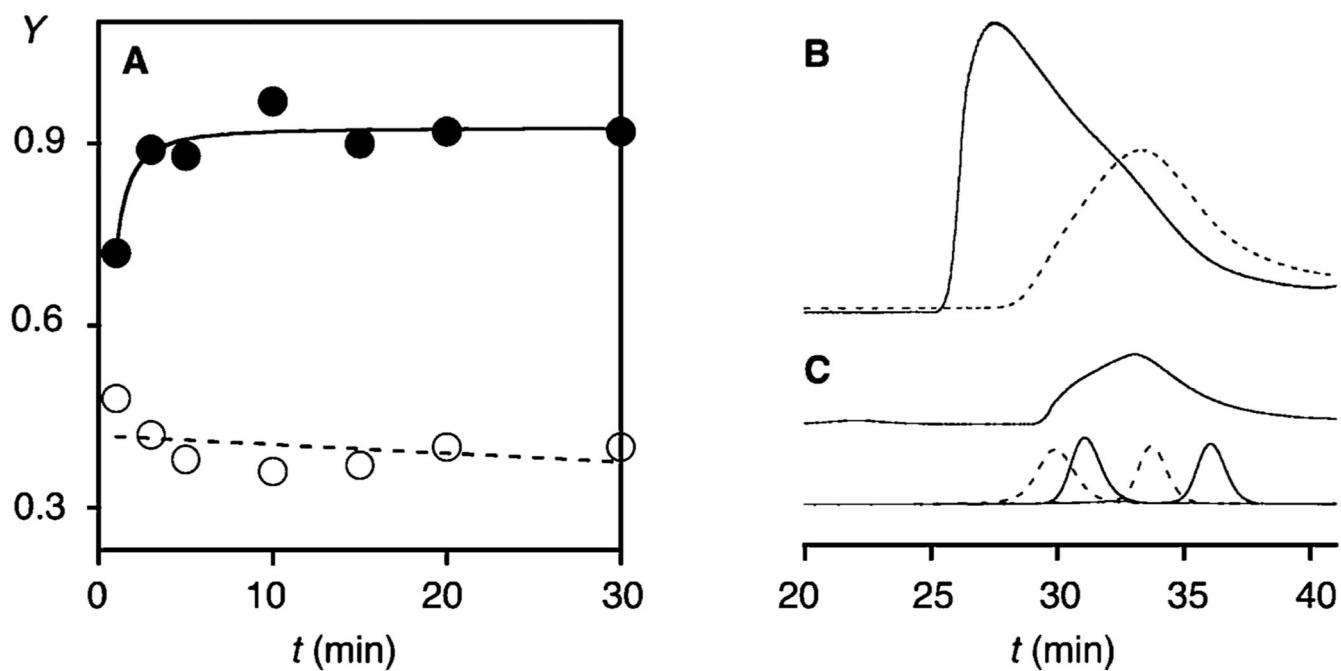
Figure 3.
Polymers of 1 made with (●, solid) and without (○, dashed) initiator 5. (A) *Y* during polymerization of 1 (100 mM) with (●) and without 5 (○, 5 mM, pH 7). (B) GPC of 1 (100 mM) polymerized with (solid) and without 5 (dashed, 5 mM, pH 7), compared to (C) polyarginine (top) and standards (bottom, M_W 43, 25, 13.7, and 6.5 kDa); Superdex 75, 30% acetonitrile in acetate buffer, pH 6.5.
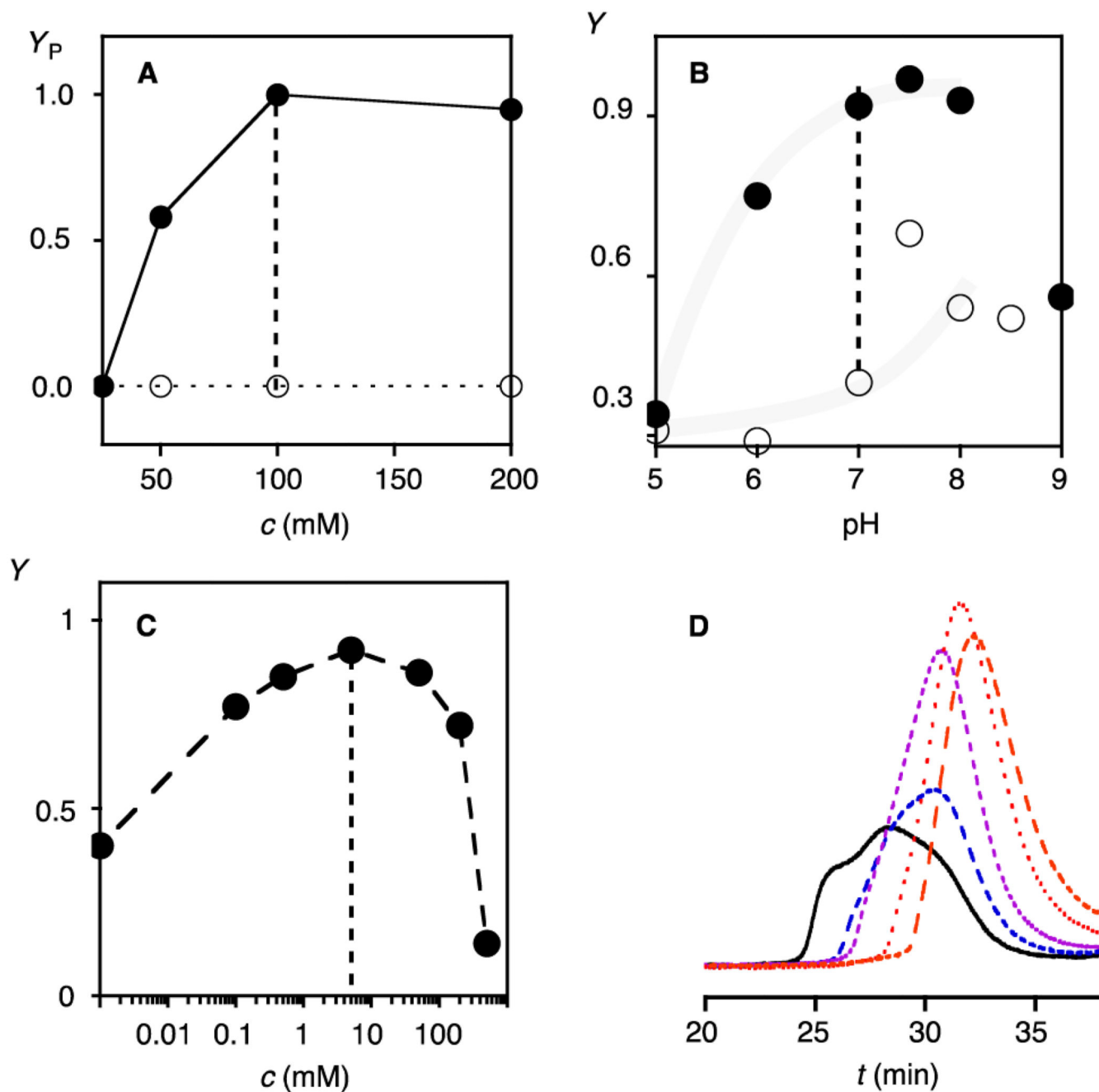
Figure 4.
Dependence on propagator concentration (A), pH (B) and initiator concentration (C, D). (A) Y_P after polymerization of increasing concentration c of 1 with (●) and without 5 (○, 5 mM, 1 M TEOA, pH 7, 10 min; assay: 75 μM guanidinium each, $Y_P = Y$ normalized to $Y = 0$ before polymerization and $Y = 1$ for maximal activity). (B) Y after polymerization with increasing pH (5 mM 5 (●), 0 mM 5 (○), 100 mM 1, 1 M buffer).[7] (C) Y after polymerization with increasing concentration of 5 (100 mM 1, pH 7). (D) GPC after polymerization with 5 (with increasing t_R: 0.5, 1, 2, 5, 10 mM; 200 mM 2, pH 7.5).
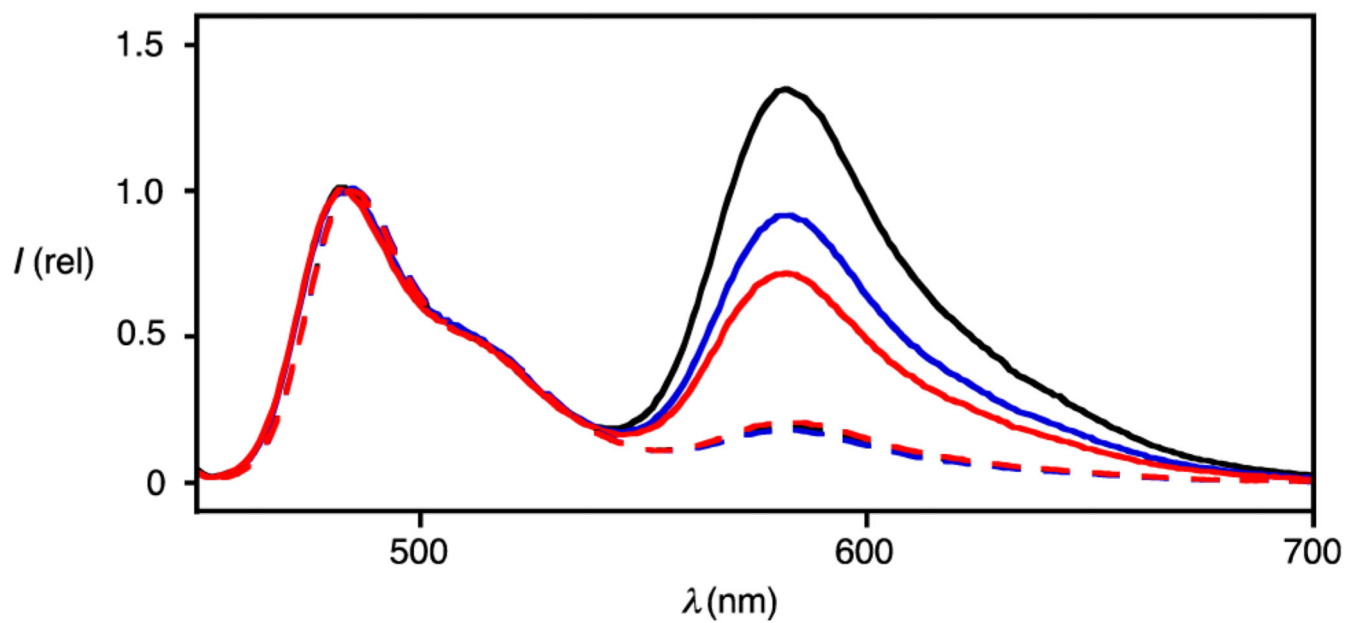
Figure 5.
FRET from initiator 7 to terminator 8. Emission spectra (λ_{ex} = 445 nm, CHCl$_3$) after polymerization of 4 (25 mM) with 7 (1 mM) for 5 s (black), 30 s (blue) and 60 s (red) in CHCl$_3$/DMF 3:1 (0.25% DIEA), terminated with 8 (2 mM, solid) and depolymerized with DTT (10 mM, dashed).