Polydiacetylene Supramolecules: Synthesis, Characterization, and Emerging Applications

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Abstract: Polydiacetylenes are a class of polymers with unique optical properties. Upon photopolymerization, monomers form a deep blue, non-fluorescent polymer, which transitions to a red, fluorescent polymer in response to various environmental factors, such as pH, temperature, or molecular binding. The chromatic and emissive properties of polydiacetylenes have generated considerable popularity for their use in biosensing applications over the past three decades. The versatility of polydiacetylene forms has also allowed for a wide range of sensors, such as: liposome bacterial sensors, films for detecting influenza virus, hydrogels for protein detection, and printed ink for the detection of volatile organic compounds. In this article, we review the wide range of techniques employed in the development of polydiacetylene sensors and summarize methods to modify, characterize, and analyze polydiacetylene-based sensing systems. Additionally, we discuss the recent directions of polydiacetylene materials outside of sensing applications as versatile tools in biomedicine and tissue engineering.

1. INTRODUCTION

Polydiacetylenes (PDAs) are a class of polymers with highly conjugated backbones that have become increasingly attractive as biosensing materials due to their unique chromatic and emissive properties. PDA materials provide a number of advantages attributable to their ease of formation under self-assembly systems. The polymerization of diacetylenes (DA) to form PDA commonly occurs via photoreaction without the need for chemical initiators, which enables high purity synthesis with no by-products. The chromatic properties of PDA arise from its highly structured alternating ene–yne backbone. Upon UV or γ- irradiation, monomers polymerize to appear optically blue and non-fluorescent. Exposing blue PDA to environmental stimuli such as changes in pH,[1, 2] temperature (thermochromism),[3-5] mechanical stress (mechanochromism),[6, 7] and molecular binding events (affinochromism/biochromism),[8, 9] results in a shift in its absorption spectrum from low to high energy, resulting in the appearance of optically red PDA that also exhibits red fluorescence.

The specific biochromism demonstrated by PDA is due to their chemical adaptability. In particular, the pendant side-chains on the hydrophilic heads of DA monomers are easily modified to facilitate their interaction with target molecules. Specifically, conjugating functional detection probes onto these head groups can generate chromatic PDA signals in response to specific biomolecule detection. PDA systems conjugated with antibodies,[10-13] aptamers,[14-17] proteins,[12, 18, 19] haptens,[20] and various functional groups[21, 22] have been demonstrated. Due to their built-in chromatic conversion, PDA platforms forego the need for costly secondary labels and detection instruments. This extensive adaptability of PDA furthers its potential in label-free biosensing systems.

Since the initial development of PDA films by Wegner in 1969,[23] PDA materials have been prepared in a variety of forms and composed as mixtures with other lipid constituents.[24] Currently, PDA is most commonly synthesized as self-assembled bilayer liposomes in aqueous solutions;[13, 21, 25-27] however, recent developments in PDA biosensors have given rise to the immobilization of PDA liposomes on solid substrates, such as microbeads[28] and cellulose membranes.[29, 30] More recent applications have employed PDA materials with host matrices, including sol-gel materials,[10, 31-33] fibrous scaffolds,[34] graphene sheets,[35] and polyvinylidene fluoride membranes.[12, 36, 37,38] The use of fluorescent red-phase PDA has also been explored in highly sensitive microarray assays and microfluidic chip sensors.[15, 39] The properties and applications of PDA materials have been reviewed and discussed over the years.[39-47,48,49,50] However, as PDA technology progresses, researchers continue to establish a wide variety of methods to synthesize and design PDA platforms, and there is a need to tabulate these approaches to better facilitate advancement and prepare new researchers interested in PDA technologies. In this strategic review of PDA materials, we discuss the fundamental properties of PDA and organize the diverse methods of PDA synthesis. This includes compiling the various approaches to conjugating

functional materials onto PDA and the role of adding external constituents into PDA materials. We will also summarize current techniques used to characterize and quantify color change in PDA sensors. Finally, we will review some of the most recent platforms for PDA systems. This review is targeted for researchers of all scientific backgrounds as both an introduction to and an update on the current state of PDA materials. Due to previous in-depth discussion of PDA sensors as Langmuir-Blodgett (LB) films, [44] these will not be discussed in detail in this article.

2. POLYDIACETYLENES

2.1 Brief History

The first investigation into PDA materials was carried out by Wegner in 1969.[23] In his initial studies Wegner achieved photopolymerization of bis-(*p*-toluene sulfonate) to create purple crystals that transitioned to red after UV polymerization.[51] The application of PDA materials as biosensors was pioneered largely by Charych and colleagues in the early 1990's.[9, 43, 52-56] Most notably, Charych and colleagues demonstrated the blue to red color transitions of PDA film in response to the binding of influenza virus on PDA films covalently modified with sialic acid.[9] These early investigations into PDA for biosensing materials resulted in the synthesis of a number of chromatic DAs ranging in color from blue to orange; however 10, 12-pentacosadiynoic acid (PCDA) and 10, 12-tricosadiynoic acid (TCDA) were favored by researchers for their stable blue to red color transitions in response to molecular binding events[43] (Figure 1A). PCDA and TCDA are currently the most widely used in PDA applications and are commercially available as off-the-shelf compounds.

2.2 Properties of Polydiacetylenes

PDA is photosynthesized via 1,4 addition of the diacetylenic moiety of DA monomers by UV or γ- irradiation (Figure 1B). Due to their amphiphilic structure, DA monomers will naturally align in aqueous solution as a result of van der Waals' interactions between the hydrophobic alkyl chains.[40, 57] This is favorable because proper packing and alignment of the DA moiety is required for polymerization to occur.[58-60] Upon 254 nm UV irradiation, PDA supramolecules appear deep blue (A_{max} ~640 nm). The appearance of color in polymerized PDA materials is a result of optical π-π* absorption in the alternating ene-yne conjugated polymer backbone.[40, 61] The exertion of external stimuli to the backbone causes an irreversible shift in absorbance from low to high energy, resulting in the appearance of red-phase PDA (A_{max} ~540 nm).[40]

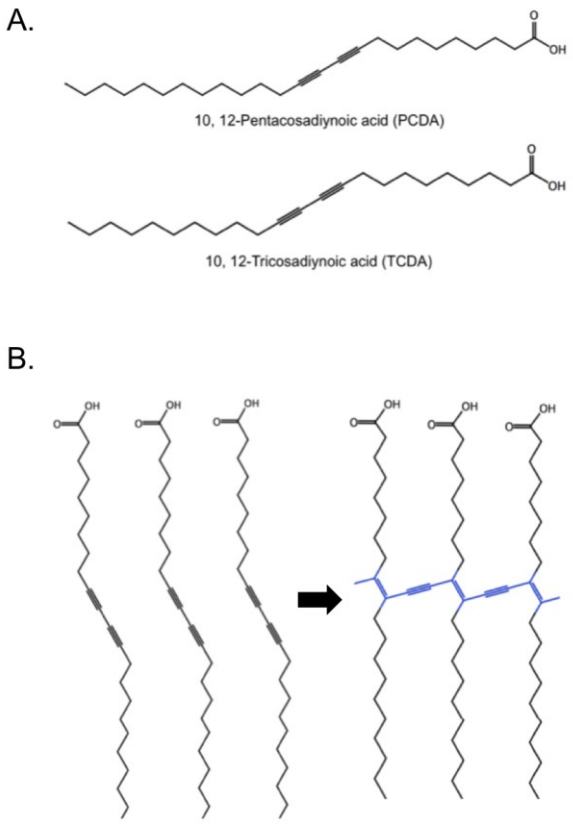


Figure 1. Molecular structure of diacetylenes. (A) Structure of commercially available diacetylenes PCDA and TCDA. (B) polymerization of diacetylenes via 1,4 addition.

The mechanism of transition between the blue-phase and red-phase PDA remains to be fully elucidated. It is proposed that after photopolymerization, the resulting blue-phase PDA backbone is prevented from adopting a fully relaxed configuration due to geometric restrictions imposed by the head groups. The exertion of environmental stimuli causes fluctuations in the head group configuration thereby disrupting the planarity of the conjugated backbone via rotational changes about the C-C bonds[40, 44] (Figure 2). This shifts the π-orbital overlaps causing a blue-shift in the absorption spectrum of the PDA backbone.

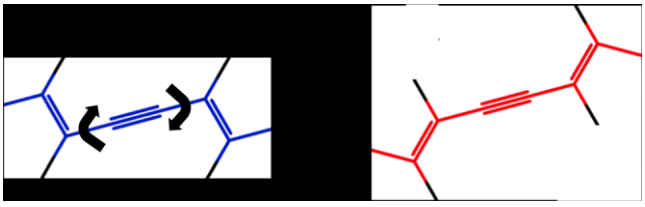


Figure 2. Slight rotations in the PDA backbone result in the conversion from blue phase to red phase.

Red-phase PDA is perceived to be more thermodynamically stable than blue-phase PDA, causing the irreversible character of PDA transitions.[40, 62-64] This mechanism of transition is supported by the observation of engineered reversible PDA materials, in which the DA head groups are modified to include amide, aromatic, and carboxylic acid groups. Such

modifications enable stable H-bonding between head groups in the initial blue-phase configuration and throughout treatment, allowing the PDA backbone to return to blue-phase planarity upon release of external stimuli (e.g. heat and pH treatment).[62, 65, 66] While the development of reversible PDA materials is another desirable aspect for biosensing solutions, this review will focus on irreversible PDA materials.

Red-phase PDA exhibits intense fluorescence properties that are not observed in blue-phase PDA. The reason for this is suggested to be an energy shift in the lowest excited state from the blue-phase to the red-phase. In the blue-phase, the lowest excited state exhibits A_g symmetry, a dipole-forbidden transition. In the red-phase, the lowest excited state exhibits B_u symmetry, which allows for radiative decay resulting in fluorescence.[40, 67, 68] Red-phase PDA will emit red fluorescence with emission peaks at approximately 560 and 640 nm when excited with wavelengths at 450 nm and above.[69]

The unique chromogenic and fluorogenic properties of PDA make it a desirable material for biosensing applications. Specifically, their internal "switch" to give a visual output via blue to red color transitions in response to external stimuli makes PDA materials especially attractive for user-friendly, in-field, diagnostic applications. This characteristic of PDA enables the production of one-step detection devices, foregoing the need for secondary labels to visualize analyte detection. The additional property of PDA materials to transition from a non-fluorescent to fluorescence-emitting state further lends potential for the development of highly sensitive sensors[15, 70] and possibilities for signal amplification, for example, via Förster resonance energy transfer (FRET) applications.[71, 72]

3. SYNTHESIS AND MODIFICATION OF POLYDIACETYLENES FOR BIODETECTION

PDA polymers are commonly synthesized from commercially available PCDA and TCDA monomers. In the advancement of PDA technologies, researchers have explored a vast range of methods to tailor PDA materials and enhance their properties. A few examples include approaches to increase their detection sensitivity, biomimetic properties (for PDA liposomes), and to incorporate PDA into various material forms. In this section, we discuss the methods by which researchers have tailored PDA materials for biosensing. For a detailed discussion of the synthesis of PDA films and liposomes, readers are referred to a comprehensive review by Reppy and Pindzola.[44] The present article instead focuses on PDA modifications to enhance biodetection.

3.1 Synthesis of Polydiacetylene Liposomes

Many recent advancements involve the manipulation of PDA liposomes that are synthesized in colloidal suspension. Due to their amphiphilic nature, DA monomers will form self-assembled bilayer liposomes in aqueous solution. A general procedure for the preparation of PDA liposomes involves first dissolving the DA monomers, along with any other lipid constituents desired, in chloroform to create an even distribution of monomers. If no additives are desired or only one type of DA monomer is being used, this step may be omitted. Chloroform is then evaporated, often in vacuum, by N_2 stream, or via rotary evaporation. The resulting mixture is resuspended in deionized water or aqueous buffer and dispersed by sonication at above 60°C, the phase transition temperature (T_m) of DAs. The resulting solution is commonly filtered through a porous membrane to remove any aggregates. In order to promote liposome stability and alignment of the DA backbone, the colloidal suspension is stored at 4°C for at least 4 h. The transparent solution is then irradiated under 254 nm UV light resulting in the appearance of a blue PDA polymer solution. PDA liposome solutions commonly range from 0.5 to 2 mM total lipid concentration, as higher concentrations can cause the PDA to precipitate out of solution.[44]

3.2 Modifications of Polydiacetylenes for Biodetection

While PDA materials are naturally responsive to environmental stimuli, researchers have explored various modifications in order to adapt and enhance the polymer for specific detection of biomolecules (Table 1). Here, we review a number of these approaches.

3.2.1 Lipid Doping

The addition of other lipids, most commonly phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), to DA mixtures to form phospholipid/PDA liposomes was first demonstrated by Jelinek and colleagues in the early 2000s.[25, 73-77] In such composite liposomes, DMPC was found to form sub-domains within the PDA membrane and did not inhibit polymerization. While initial investigations of DMPC/PDA liposomes were for their biomimetic properties in studies involving cell membrane interactions,[26] subsequent reports highlighted their use in biodetection. Taking advantage of hydrophobic ionophores with specific ion selectivity, Jelinek and colleagues successfully incorporated ionophores into the phospholipid domain of the of the DMPC/PDA liposomes. Subsequent addition of ions to DMPC/PDA liposome solutions resulted in specific interactions between ions and ionophores causing a color response visible to the naked eye.[75]

Jelinek and colleagues further demonstrated the feasibility of this method through reports of specific interactions between antibodies with epitopes embedded into phospholipid domains of DMPC/PDA liposomes,[74] and again with specific protein interactions with embedded calixarenes[24] (Figure 3). Notably, in these studies, the embedding of detection biomolecules into the phospholipid domain of liposomes occurred after polymerization, but did not result in significant color transitions. The same group also employed unmodified DMPC/PDA composites as bacterial sensors.[78, 79] The design of these sensors benefits from the interaction between

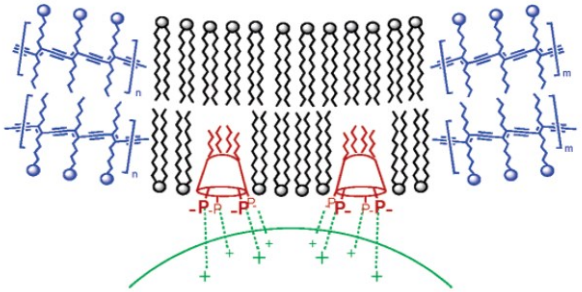


Figure 3. Phospholipid doping of PDA results in a mixed liposome membrane composed of phospholipid (black) and PDA (blue) subdomains. The embedding of membrane proteins into the phospholipid domains can act as receptors for target analytes. Reproduced with permission from ref [24]. 2006, Copyright (2006) *American Chemical Society.*

bacterial endotoxins and membrane phospholipids. Although this non-specific approach cannot distinguish detection of specific bacterial strains, color transitions in the liposome-laden agar scaffolds were observed in the presence of both gram-negative and gram-positive bacteria.[79]

Additional investigations into DMPC/PDA liposomes suggest that they enhance the properties of PDA sensors.[13, 80] Su et al.[13] reported that 40:60 DMPC:PDA liposomes exhibit up to 35% higher sensitivity of antigen detection by anti-human immunoglobulin (h-IgG) antibody-conjugated PDA as compared to liposomes containing 0% DMPC, as determined by percent Color Response (CR) (Section 4.2.1). Similarly, a report by Kim et al.[80] found that liposomes composed of 20-40% DMPC displayed expedited color transitions as compared to lower DMPC percentages in the detection of *E. coli*. Notably, both studies indicate that liposome morphology at 40% DMPC resembles flat sheets rather than circular liposomes (Figure 4). Studies investigating the morphological changes of 4:6 DMPC/PDA materials have reported varying morphologies, including circular liposomes, flat sheets, and fibers.[13, 44, 53, 81] A discussion of these is available elsewhere.[44]

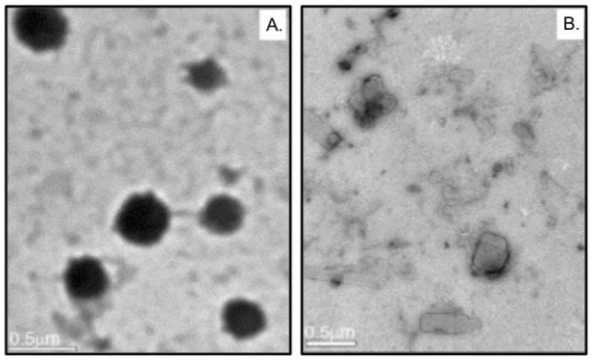


Figure 4. TEM images of DMPC/PDA with 0% DMPC (A) and 40% DMPC (B). Labels recreated for consistency. Reproduced with permission from ref [13]. Copyright 2004, *Elsevier B.V.*

Table 1. Modifications and Methods to Characterize PDA sensors

Ref.	Composition	Target	Detection Limit	Characterization	Naked Eye
[11]	PCDA and PCDA-Maleimide liposomes	*C. parvum*	10^6 oocysts/mL	^{1}H NMR, spectrophotometry (UV-Vis), differential scanning calorimetry (DSC), transmission electron microscopy (TEM)	Yes
[14]	PCDA and TCDA liposomes doped with DMPE	Thrombin	0.5 mM thrombin	Scanning electron microscopy (SEM), fourier transfrom infrared spectroscopy (FTIR), dynamic light scattering (DLS), spectrophotometry (UV-Vis)	Yes
[19]	Mixed DA liposomes	Bacterial lipopolysaccharide (LPS)	4.91 x 10^8 cells/mL *E. coli*	DLS, atomic force microscopy (AFM), spectrophotometry-(UV-Vis, fluorescence)	No
[82]	PCDA liposomes with alkyl chain spacers	Diethyl phosphate (DEP)	N/A (multiple targets were tested, all at 1 mM)	Spectrophotometry (UV-Vis, photoluminescence)	N/A
[83]	DA-2,2'-(ethylenedioxy)bis(ethylamine) (DA-EDEA) liposomes	Heparin	2.5 μM in buffer, 5.6 μM in serum	DLS, Spectrophotometry (UV-Vis)	No

16	PCDA-NHS liposomes with carboxylic acid spacers	Potassium	0.1 mM potassium	Spectrophotometry (UV-Vis, photoluminescence)	No
10	TCDA-NHS liposomes doped with DMPC in hydrogel beads	Phosphinothricin acetyltransferase (PAT)	20 nM PAT	Spectrophotometry (UV-Vis)	Yes
12	TCDA-NH_2/TCDA-OH; TCDA-OH/ Diamine	Streptavidin (STA); microalbuminuria (MAU)	170 nM STA; 2 μg/mL MAU	FTIR, Spectrophotometry (UV-Vis, photoluminescence)	No
84	Organic solvent-modified PDA ink emulsion printed on paper	Bisurethane	Discrimination between tetrahydrofuran, methylene chloride and chloroform was achieved	Spectrophotometry (UV-Vis), fluorescence imaging	Yes
85	Intercalator-modified PCDA liposomes	Double-stranded DNA amplified from genomic DNA	20 nM of DNA with a length of ~100 base pairs	Spectrophotometry (UV-Vis), TEM	Yes

The mechanism by which DMPC doping increases sensitivity of PDA materials is unknown and has yet to be fully investigated. A number of reports indicated an increase in flexibility of phospholipid/PDA composite materials through interruptions in the rigid PDA backbone.[26, 76, 77, 86] It is proposed that the insertion of DMPC along the PDA backbone disrupts hydrogen bonding between PDA head groups. This results in a less rigid PDA structure, which likely enables more sensitive blue to red transitions from biomolecular recognition events.[13, 80]

While DMPC remains the most widely used dopant in PDA composite materials, several studies further explored additional phospholipids such as 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE)[14, 87-90] and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG),[87-90] among others.[78, 91, 92] Whereas a number of recent studies incorporated such additional phospholipids to increase the biomimetic properties of PDA liposome membranes for the purpose of studying cell membrane interactions,[87, 89, 91] Deming and colleagues have highlighted the preferential interaction of certain amino acids for particular phospholipids. For example, Lysine and Leucine induced more sensitive color transitions when exposed to DMPG/PDA and DMPE/PDA liposomes as compared to DMPC/PDA.[90] Similarly, Jelinek and colleagues have recently determined lipid-dependent sensitivities in PDA fingerprinting platforms for bacterial detection. Specifically, films made with three different lipids DMPC/PDA, 1-α-dioleoylphosphatidylethanolamine (DOPE)/PDA, and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG)/PDA each resulted in a distinct color transition when exposed to certain strains of bacteria (Figure 5).[78] Kang et al. demonstrated detection of aminoglycosidic antibiotics using PDA- phospha- tidylinositol-4,5-bisphosphate (PDA-PIP_2).[93] The PDA-PIP_2 liposomes showed highest specificity when spiked with 1,2-Dimyristoyl-sn-glycero-3-phosphate (DPMA), as it increases the mobility of the PDA backbone.[93] Such findings suggest the significance of the dopant lipid head groups in biodetection assays and indicate yet another approach to tailor PDA materials for more specific and sensitive biomolecular interactions.

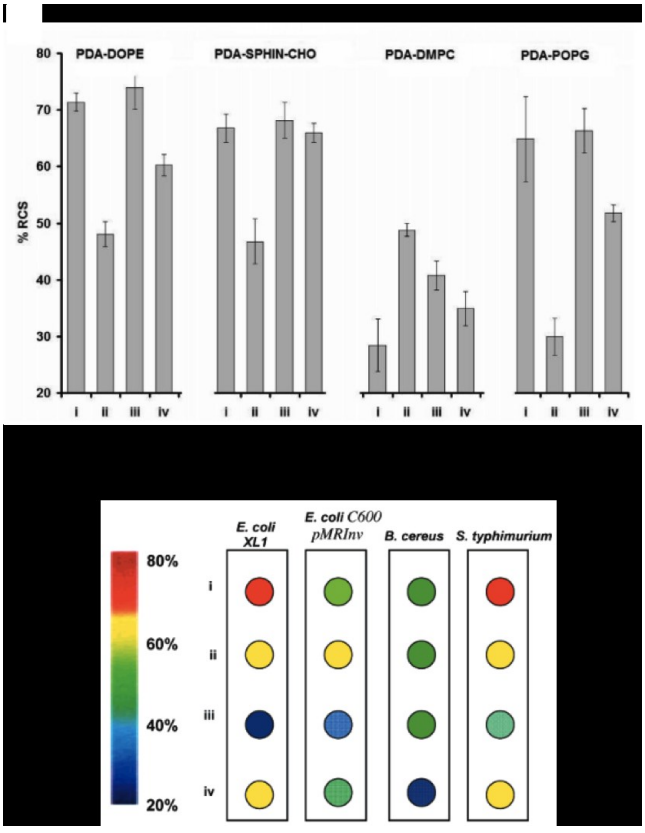


Figure 5. PDA fingerprinting of bacterial strains. Four types of PDA films created with varying lipid dopants exhibit unique color transitions when exposed to different strains of bacteria. Adapted from ref [78]. Copyright 2007, *American Chemical Society*.

3. 2.2 Covalent Modifications

PDA conjugates have been investigated for biomolecular detection since initial studies by Charych and colleagues, in which the modification of DA monomers with sialic acid via carboxylic ester synthesis was reported in the detection of the influenza virus.[9] In this subsection, we discuss the general techniques used to conjugate functional biomaterials to PDA and review a number of strategic approaches to increase detection sensitivity through covalent modifications.

3.2.2.1 PDA intermediates. The modification of PDA materials often refers to the conjugation of functional compounds onto the hydrophilic head groups of PCDA and TCDA monomers. Specifically, carboxylic ester synthesis from the carboxylic acid moiety of the DA monomer and an amine group on the biodetection probe (e.g. antibody, aptamer, functional group, etc.) occurs via activation of the carboxylic acid with N-hydroxysuccinimide (NHS) and ethyl(dimethylaminopropyl) carbodiimide (EDC).[94] While the conjugation of biodetection probes can follow immediately

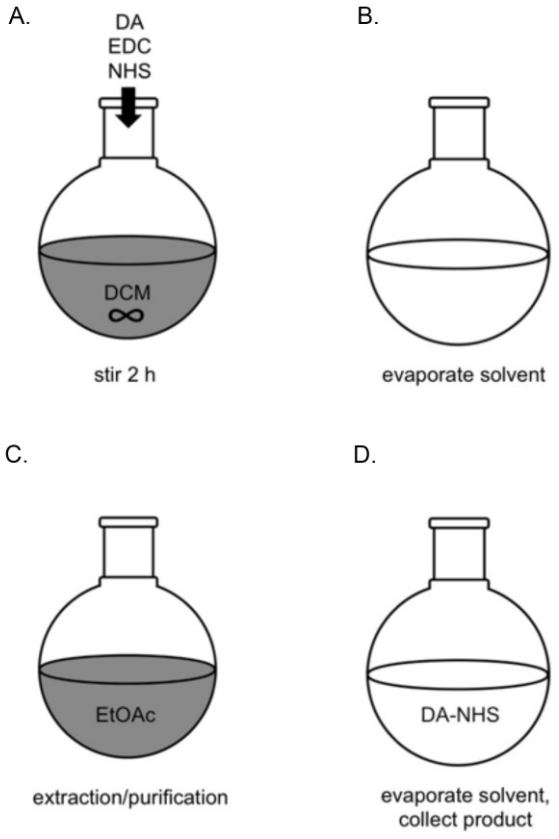


Figure 6. Protocol for conversion of diacetylenes (DA) to their succinimide ester (DA-NHS). (A) Stir a mixture of DA, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide-hydrochloride (EDC-HCl) and N-hydroxysuccinimide (NHS) in dichloromethane (DCM) for 2 h. (B) Evaporate the solvent (dichloromethane) and (C) purify the DA-NHS by resuspending in ethyl acetate (EtOAc). (D) Evaporate the solvent (ethyl acetate) and collect the product.

after the conversion of DA monomers to the succinimidyl ester, an alternative is to synthesize and store the activated DA intermediate, DA-NHS, for further use. A general procedure for the synthesis of DA-NHS involves reaction of the DA monomers with excess NHS and EDC in methylene chloride for 2 hours at room temperature. Next, removal of the organic solvent (dichloromethane) by evaporation is followed by extraction using ethyl acetate. The resulting DA-NHS monomer is a white solid (Figure 6).

More recently, Kim and colleagues have reported studies incorporating the addition of linker or spacer molecules to DA intermediates.[15, 16, 95] In these reports, 2,2'-(ethylenedioxy)bis(ethylamine) (EDEA)[16] and 2-(2-(2-Bromoethoxy)ethoxy)ethanol[95] were conjugated after the synthesis of DA-NHS and before the conjugation of biodetection probes. Interestingly, further investigation by Kim and colleagues into the effect of alkyl spacer moieties on color transformations of PDA materials demonstrated that the sensitivity of color transitions decreased with increasing alkyl spacer length (Figure 7).[82] This result is supported by the current proposed mechanism of color transitions (Section 2.2), in which increasing alkyl spacer lengths is associated with an increase in van der Waals' interactions between monomer side-chains, thereby stabilizing the blue phase through restricting rotations in the PDA backbone. Similarly, the length of the alkyl chain that comprise the hydrophobic tail of the DA monomer influences sensitivity. In a recent study by Park et al., PDA sensors, fabricated by coating monomers with varying sizes of alkyl chains (C18-C25) on porous silica plates, exhibited varying sensitivity when exposed to ammonia gas.[96] Red fluorescence intensity of 10,12-octadecadiynoic acid (C18)-coated sensors were approximately 20 times greater than that of TCDA (C23)-coated sensors.[96] These studies indicate that the composition of spacer, linker, and alkyl chain

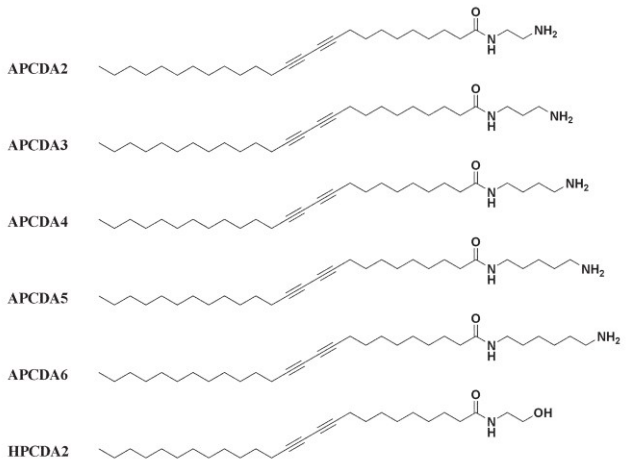


Figure 7. PCDA monomers modified with varying linker lengths from 2 to 6 carbons. Reproduced with permission from ref [82]. Copyright 2010, *John Wiley & Sons, Inc*.

moieties have significant effects on detection sensitivity, however, more investigation is needed in this area.

Kim and colleagues have further explored an alternative to DA-NHS intermediates by investigating DA-epoxy conjugates due to the known stability of epoxy groups under proper storage and their ability to readily react with amine groups.[15, 70, 95] Accordingly, their results suggest that DA-epoxy provides higher stability as compared to DA-NHS intermediates.

The reports from such explorations into the effects of additional linkers between conjugated detection probes and the PDA membrane surface as well as the use of alternative intermediates for probe conjugation are significant for both increasing detection sensitivities and revealing properties behind PDA color transformations.

3.2.2.2 Functional materials. The functionalization of DA monomers with biomolecular detection probes including antibodies,[10-13] aptamers,[14-17] proteins,[12, 18, 19] and others[8, 21, 22, 83, 97-99] have been reported. Generally, these involve the spontaneous reaction of DA-NHS esters with an amine group on the detection probe in aqueous solution, followed by a step to remove unreacted molecules. Methods of conjugation vary at the time point in which conjugation occurs. For example, in a study by Jung et al.,[14] thrombin aptamers were conjugated to previously prepared, unpolymerized DA-NHS monomers in excess, and unreacted monomers were removed by centrifugal filtration. DA monomers functionalized with thrombin aptamer were then mixed with non-functionalized lipids for PDA liposome formation. Alternatively, another approach by the same group[10] converted unpolymerized DA to succinimidyl esters as self-assembled liposomes and removed unreacted NHS by centrifugation. Anti-phosphinothricin acetyltransferase (PAT) antibodies were then reacted with the DA-NHS liposomes in solution. Unconjugated antibodies were removed via centrifugal filtration and unreacted esters were quenched with ethanolamine. Another study by Lee et al.[16] described the conjugation of potassium aptamers in excess onto unpolymerized, self-assembled DA-NHS liposomes. Unbound aptamers were then removed by dialysis prior to photopolymerization.

The percent of probe-conjugated DAs (i.e., the ratio of probe-conjugated DAs to total lipids) is significant but not well understood. In early investigations, Charych and colleagues determined that for optimal detection of influenza virus, PDA film sensors were to be fabricated with 5-10% sialic acid-conjugated PDA.[25, 52] This is additionally reflected in Jung et al.,[14] in which the optimal percentage of thrombin aptamer-conjugated PDA was reported at 6.7%. Contrastingly, Park et al.[12] indicated that optical transformations were most sensitive to streptavidin when biotin-conjugated PDA monomers were at 20-40%. Alternatively, Lee et al.[15] reported the fabrication of mercury(II) sensors with 80% of aptamer-conjugated PDA. These varied findings indicate that more investigation is needed to elucidate the relationship between percentage of probe-conjugated PDA and sensor sensitivity.

The conjugation of functional groups to PDA head groups has also been investigated as a method to enhance biodetection. Specifically, amine-terminated (-NH_2) PDA materials have been reported for detection of negatively charged biomolecules.[22, 83, 97, 98] Alternatively, Chung and colleagues have investigated the use of functional groups to prevent non-specific binding.[12] In their report, amine-terminated, hydroxyl-terminated (-OH), and naturally occurring carboxylic acid-terminated (e.g. TCDA) PDA were studied for non-specific interactions with bovine serum albumin (BSA). They showed that PDA composed of TCDA-OH exhibited the least amount of non-specific interactions with BSA due to the electrically neutral and hydrophilic properties of the hydroxyl functional group in phosphate buffered saline (PBS, pH = 7.4). This report indicates an approach to enhance the signal to noise ratio of PDA biosensors by minimizing non-specific binding.

4. CHARACTERIZATION OF POLYDIACETYLENES

4.1 Properties

Following the synthesis of PDA materials, it is necessary to determine the basic properties of the synthesized polymer in order to confirm successful conjugations. In this subsection we discuss a few of the methods currently employed to characterize PDA materials.

4.1.1 Color

In order to characterize the color properties of PDA, it is common to measure the absorbance spectra of the material in the visible spectrum (Figure 8A). Specifically, UV-Vis spectroscopy measurements are done using a spectrometer. Blue-phase PDA are associated with an absorbance maximum at approximately 640 nm and a vibronic shoulder peak at 600 nm.[13, 40] Alternatively, red-phase PDA are characterized by a shift in absorbance peak to approximately 540 nm.[40] The intermediate purple phase, distinct from a mixture of blue- and red-phase PDA, is associated with a peak at approximately 600 nm. It is speculated that this peak is related to the secondary absorbance peak associated with blue-phase PDA at the same wavelength.[31, 43, 44, 100-102] The peak absorbance values are commonly used to quantify blue to red transitions of PDA materials through the colorimetric response (CR) value (Section 4.2.1).

Due to the intense fluorogenic properties of red-phase PDA (Figure 8B), another way to contrast the blue and red phase is through measuring the fluorescence spectra using a fluorometer. The red phase is characterized by emission peaks at 560 and 640 nm (Figure 8C), while the blue phase exhibits no fluorescence properties.[69] Accordingly, measuring photoluminescence or fluorescence intensity of the material is yet another option (Figure 8D).[16] As a result, it is possible to indirectly quantify blue to red color transitions as a

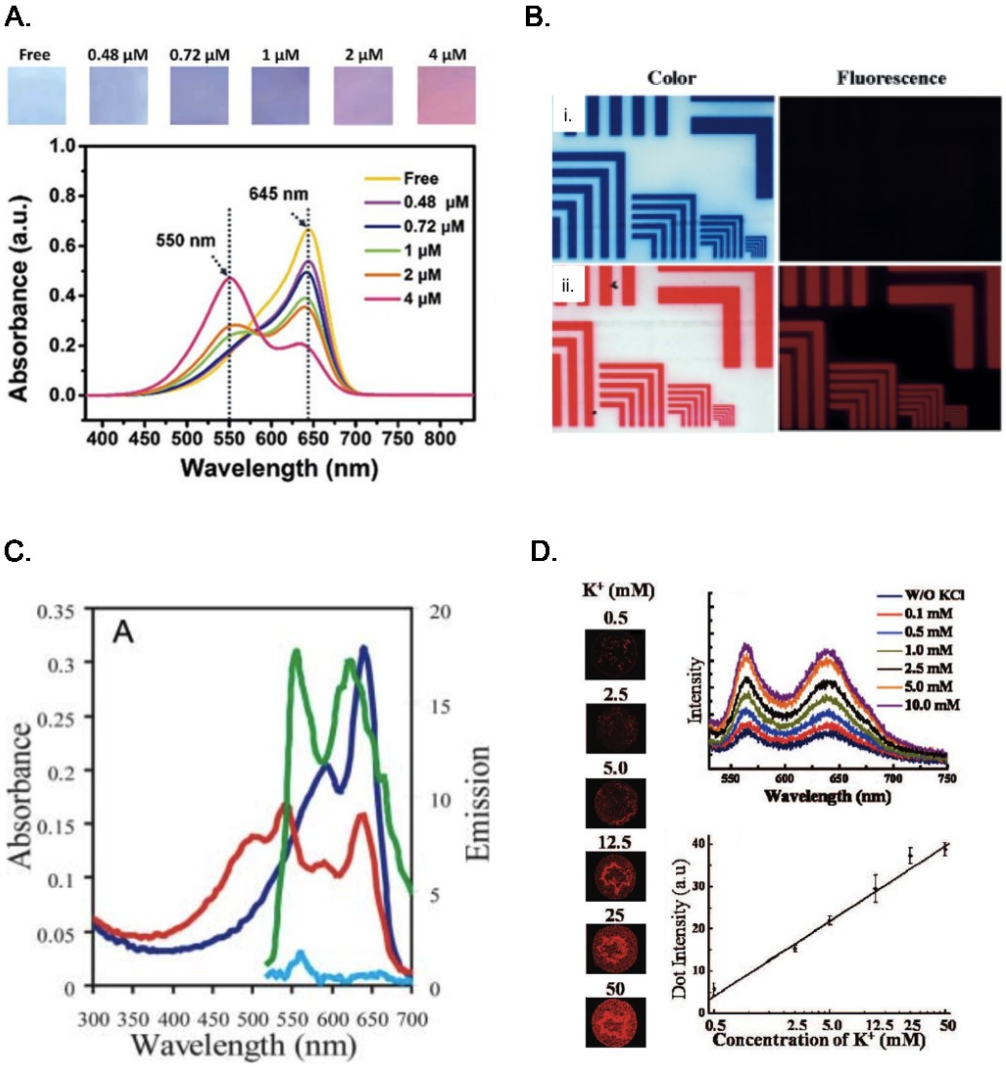


Figure 8. Color properties of PDA. (A) UV-Vis spectra of PDA strips exhibiting various colors from blue to red. Adapted ref [103], Copyright 2014, *Royal Society of Chemistry*. (B) Color and fluorescence images of blue phase and red phase PDA. Adapted from ref [65], Copyright 2006, *John Wiley & Sons, Inc.* (C) Absorbance spectra of blue phase (blue) and red phase (red) PDA. Emission spectra of blue phase (light blue) and red phase (green) PDA. Adapted from ref [44], Copyright 2007, *Royal Society of Chemistry*. (D) Fluorescence images (left), spectra (top right), and intensity (bottom right) of red to blue phase PDA (color not shown) exhibiting varying degrees of fluorescence. Adapted from ref [16], Copyright 2008, *American Chemical Society*

measurement of fluorescence intensity of the material.[39, 45] The major advantage of this approach is the high sensitivity in fluorescence measurement systems, which allows for the detection of small changes in emission as compared to changes in absorbance or red-green-blue (RGB) values. However, the drawback to this methodology is that the need for such fluorescence measurement instruments may limit the application of these types of sensors in low-resource settings.

4.1.2 Size and Morphology

It is important to understand the size and morphology of synthesized PDA materials in order to confirm reproducibility as well as observe physical changes associated with molecular binding onto material surfaces and color transitions. In this subsection we review a few of the most common methods to determine PDA size and morphology.

Dynamic light scattering (DLS) is frequently employed to determine the size of synthesized liposomes, which vary with preparation methods. In particular, we have determined that liposome size is inversely correlated with sonication time in the preparation process (Figure 9). Other factors that we speculate to influence liposome size include sonication type (probe vs. bath), power (watts), and volume of sample. Such details are often overlooked but may be necessary for consistencies in reproducing PDA liposomes.

To observe the surface and shapes of PDA materials, transmission electron microscopy (TEM)[104] and scanning electron microscopy (SEM)[87] are frequently used (Figure 10). While both are sufficient for their purpose, higher resolution TEM is often preferred over SEM when observing nanoscale liposomes. SEM and TEM images are frequently viewed after

the synthesis of the PDA material and compared before and after molecular binding events. With the recent popularity of using substrates coated with PDA liposomes, atomic force microscopy (AFM) has also been employed to confirm and characterize the deposition of such materials. However, due to the destructive nature of AFM, blue to red color transformations are commonly observed as a result of the procedure.[40]

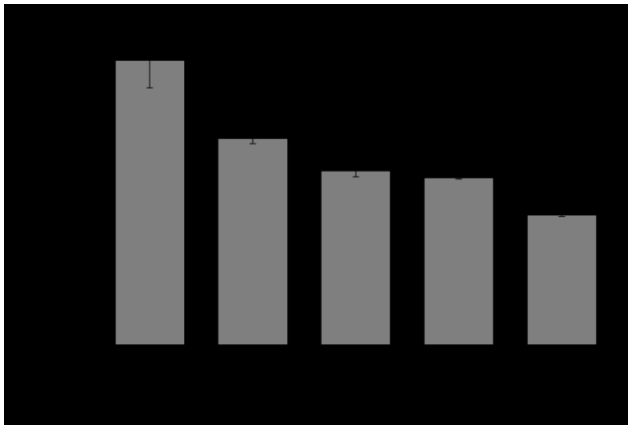


Figure 9. Size of PDA liposomes as a result of varying sonication times. Liposomes were sonicated by a probe sonicator (Qsonica Q500) at 20% amplitude. Size of liposomes was determined by DLS (Malvern Zeta90). Data are represented as mean ± SD (n = 3).

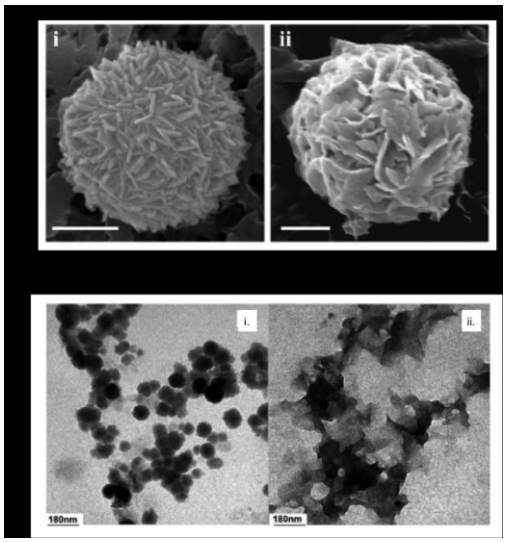
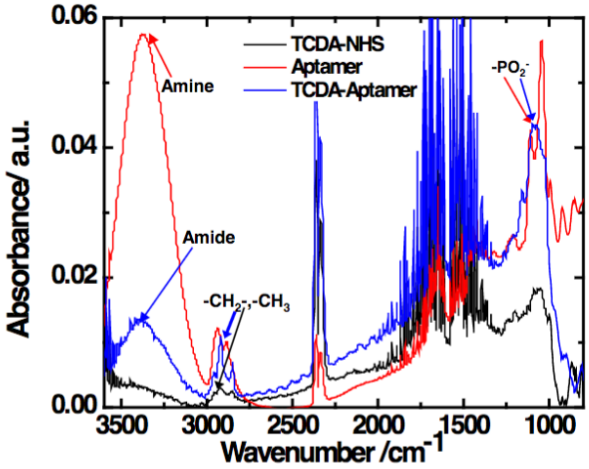


Figure 10. (A) SEM images of giant biomimetic PDA liposomes , and (B) TEM images of PB2+-sensing liposomes before (i) and after (ii) the addition of 1 μM PB^{2+} . (A) Adapted from ref [87], Copyright 2008, *John Wiley & Sons, Inc.* (B) Adapted from ref [104], Copyright 2011, *Royal Society of Chemistry.*

4.1.3 Molecular Structure

With the frequent modification and adaptation of PDA materials for specific functions, it is important to confirm successful conjugations by examining the molecular structure of synthesized PDA materials. ^{1}H nuclear magnetic resonance (NMR) is widely used to verify the synthesis of DA conjugates, in which the characteristic properties of hydrogen



atoms in response to a magnetic field can confirm the presence of specific compounds. Accordingly, PDA conjugates can be verified by comparing peaks in their ^{1}H NMR spectra.

Another approach to examine the structure of PDA materials is through Fourier transform infrared spectroscopy (FTIR), in which the presence of bonds between specific atoms can be deduced (Figure 11). While FTIR is advantageous in confirming the presence of certain compounds, it can also be used to investigate changes in the strength of hydrogen bonds

Figure 11. FTIR spectra TCDA-NHS, aminated thrombin aptamer, and TCDA conjugated with thrombin aptamer. Reproduced with permission from ref [14], Copyright 2010, *John Wiley & Sons, Inc.*

between PDA head groups.[62, 65] This is significant since intermolecular bonding between head groups is thought to play an important role in color transitions in PDA materials.

4.1.4 Other Methods

. The above described methods to characterize PDA materials are only a few of many commonly used by researchers. Other methods, including Raman spectroscopy,[105-107] differential scanning calorimetry (DSC),[105, 108, 109] and others[105, 109, 110] have been employed by a number of studies investigating PDA materials but are not discussed in this article. A thorough understanding of chemical characterization techniques would be helpful for those interested in the field of PDA biosensors.

4.2 Analysis of Optical Properties

The ability to quantify the sensitivity of a sensor is a critical aspect in evaluating its efficiency. In this subsection, we discuss current methods to quantify the optical transitions in PDA materials.

4.2.1 Colorimetric Response

The colorimetric response (CR), developed by Charych and colleagues, is the standard method of quantifying blue to red color transitions in PDA sensors.[43] In particular, the wavelength at which the absorbance maxima occur in the blue phase (~640 nm) and red phase (~540 nm) of the sensor are

subject to focus. The exact wavelengths used vary depending on the absorbance spectra of the specific PDA material being investigated. The %CR value is then determined first by calculating the percent blue, *PB*, in which:

$$PB = \frac{A_{blue}}{A_{blue} + A_{red}} \times 100\%$$

where A_{blue} is the absorbance value at the selected wavelength for the blue phase and A_{red} is the absorbance value at the selected wavelength for the red phase. From here, the %CR value is determined as:

$$CR = \frac{(PB_0 - PB_f)}{PB_0} \times 100\%$$

where PB_0 is the initial percent blue of a baseline. The advantage to this systematic approach is that any environmental factors affecting absorbance values will affect A_{blue} and A_{red} equally and will not alter overall detection. Nevertheless, with weak absorption, the CR quantification method will amplify background noise, which can reduce calculated device sensitivity[44].

4.2.2 Digital Colorimetric Analysis

In their analysis of a PDA film for the sensing of membrane-active compounds, Kolusheva, Jelinek and colleagues developed an approach that quantifies the RGB values of scanned images.[111] This quantification approach has gained recent popularity especially for analyzing fabricated PDA films.[28-30, 111-114] Volinksy et al.[111] derived this digital colorimetric analysis (DCA) method from Pratt,[115] in which the red chromaticity level, *r*, is defined as:

$$r = \frac{R}{R + G + B}$$

For PDA materials, Volinsky et al.[111] further extrapolated the equation to quantify the blue to red transitions as red chromatic shift (RCS):

$$\%\, RCS = \frac{r_{sample} - r_0}{r_{max} - r_0} \times 100\%$$

where r_{sample} is defined as the average chromaticity level from all pixels in the sample PDA image, r_0 is defined as the average red chromaticity level of a PDA image prior to color transition, and r_{max} is defined as the average red chromaticity level of a PDA image with maximum blue to red transition (i.e. positive control). Volinsky et al. further compared the results of their DCA with a parallel CR study, and indicated a close correlation between the two data sets (Figure 12). Currently, CR remains the most widely used approach to quantify blue to red color transitions of PDA materials, especially liposomes. Nevertheless, the DCA approach is advantageous in that high quality images of samples are sufficient for analysis and is an easier option for analysis of PDA films or strips.

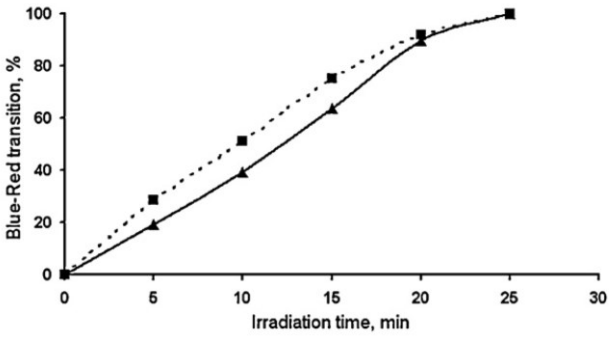


Figure 12. Blue to red transition of PDA films analyzed by digital colorimetric analysis (% RCS curve, solid line) and UV-Vis spectrophotometry (% CR curve, dashed line). Reproduced with permission from ref [111], Copyright 2007, *Elsevier B. V.*

5. RECENT ADVANCES IN PDA APPLICATIONS

The incorporation of PDA into a vast array of material forms has been previously reviewed, from PDA films and self-assembled monolayers, colloidal solutions, fluorescence microarrays, microbead coatings, sol-gel matrices, carbon nanotubes, and others.[39, 42, 44] While the bulk of this article has largely focused on PDA as biosensors, in this section, we discuss a few of the most recent and unconventional material forms currently being investigated that are not limited to the field of biosensing applications.

5.1 Paper-based sensors

One of the greatest advantages of PDA materials is for the fabrication of label-free, chromatic sensors. This is desirable because PDA platforms enable one-step, colorimetric detection of an analyte without the need for costly secondary labels. To further the advancement of low-cost PDA sensors, studies have incorporated PDA materials into paper-based sensors. These sensors are advantageous for in-field use or point-of-care applications due to their stability, ease of transport, inexpensive fabrication, and user-friendly platform without the need for external reagents or laboratory instruments. In this subsection, we review reports of paper-based PDA sensors.

Notably, Wacharasindhu and colleagues reported the specific colorimetric detection of volatile organic compounds (VOCs) using PDA-coated filter paper.[29] Specifically, eight types of PDA were tested for their sensitivity against VOC vapors, including commercially available PCDA and TCDA. Dots from unpolymerized monomer solutions of uniform size were deposited on filter paper using an automatic pipette, allowed to dry, and irradiated under 254 nm light. The PDA sensing dots were tested by attaching the coated filter paper on the inner lid of a chamber containing various VOCs. DCA of the various monomers on filter paper indicated that 6,8-nonadecadiynioic acid exhibited the greatest sensitivity to VOCs, exhibiting distinct color transitions when exposed to 18 different VOCs. This is an improvement from their previous work, reporting non-specific VOC detection.[30] Significantly,

the VOC-sensing devices were reported to be stable in a refrigerator over the span of several months.

The fabrication of paper-based, VOC-detecting PDA sensors has been further adapted in reports by Yoon et al.[116] In this investigation, VOCs both in the vapor phase and the liquid phase were detected by UV-irradiated polymers of 5,7-dodecdiyne-1,12-diol bis[((butoxycarbonyl)methyl)urethane] (4BCMU). Varied results between VOC detection from liquid and vapor exposure were observed only in tetrahydrofuran (THF), DCM (a.k.a. methylene chloride), and chloroform. Interestingly, liquid-phase detection of THF, DCM, and chloroform, along with vapor-phase detection of chloroform, resulted in blue to red to yellow color transitions that reverted to red phase upon removal from VOC. Notably when in the yellow-phase, poly-4BCMU exhibits green fluorescence (instead of red fluorescence), excitable at 488 nm (Figure 13).

Alternatively, Wang et al.[35] demonstrated the use of PDA-coated, stacked graphene film or "paper" for the detection of VOC vapors. Unmodified PCDA was used for this study. The thickness of the fabricated graphene films was reported to be ~1-40 μm and the structure was confirmed by scanning tunneling microscopy (STM). The exposure of PDA/graphene films to four VOCs gave distinct color transitions.

While early paper-based PDA sensors have generally been limited to either temperature[66, 84] or volatile compound detection,[29, 30, 35, 116] Yu and colleagues recently reported the

specific detection of Pb^{2+} ions in a paper-based assay fabricated from a nanofibrous membrane.[103, 117] In their most recent study, Yu and colleagues functionalized PCDA monomers with glycine (Gly) for the specific detection of Pb^{2+}. A polyacrylonitrile (PAN) nanofibrous membrane (NFM) was then fabricated with embedded PCDA-Gly and SiO_2 nanoparticles (NPs), in which the purpose of the SiO_2 NPs was to increase the surface area for higher exposure of PCDA-Gly to sample solutions. The structures of membranes with varying wt% from 0 to 1 wt% of SiO_2 NPs were observed with field emission SEM. Exposure of the different membranes to varying concentrations of Pb^{2+} demonstrated that PCDA-Gly PAN NFMs composed of 0.5 wt% SiO_2 NPs exhibited the highest sensitivity to low concentrations of Pb^{2+}, with a limit of detection at 0.24 μM. Currently, there are very few reports of paper-based PDA sensors. Additional developments, especially those enabling sensitive detection of targets in complex matrices (e.g., food, blood, urine, and other health-related fluids), are highly desirable.

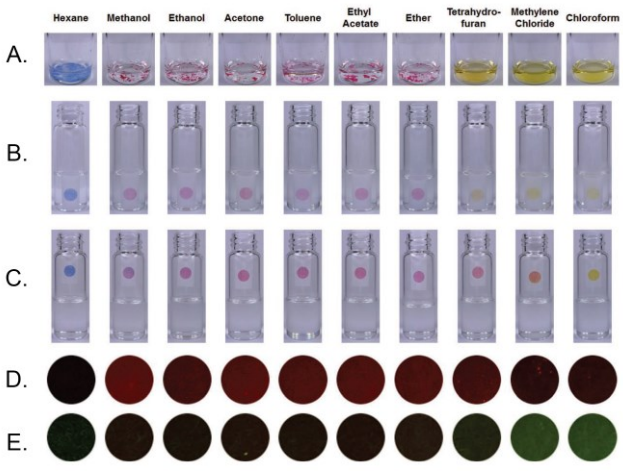


Figure 13. Color and fluorescence transitions of inkjet printed PDA (poly-4BCMU) on paper strips. Color of poly-4BCMU powder (A) and printed strips after reaction in various volatile organic compounds (B) or to their vaporized form (C). Red (excitation 546 nm, D) and green (excitation 488 nm, E) fluorescence images of poly-4BCMU strips after exposure to volatile organic compounds. Adapted from ref [84], Copyright 2013, *John Wiley & Sons, Inc.*

5.2 Drug Delivery

The advancement of drug delivery systems for anticancer therapies and their ability to selectively target tumor cells has gained popular attention over traditional non-selective treatments such as chemotherapy and radiation[118]. Due to their biocompatible properties, self-assembled liposomes composed of phospholipids have long been investigated as encapsulating agents for such drug delivery systems[119-121]. Challenges in liposome-mediated drug delivery include long-term stability of the liposome and controlled-release of the therapeutic agent. In particular, liposomes are easily disassembled in acidic/basic environments or in the presence of surfactants; additionally, liposome-cell fusion is also an area of concern.[120] Previous investigations have determined that one solution is to use polymerized liposomes, which are more stable in a range of molecular environments.[122-124]

PDA liposomes have been recently recognized in the field of drug delivery systems for their nontoxicity[125] and photo-induced polymerization without the use of toxic initiators.[47] Furthermore, the ease of chemical modification to PDA head groups along with the control over their degree of polymerization via mixed phospholipid/PDA liposomes have made PDA an attractive drug delivery system over the past decade. In this subsection, we discuss some of recent advances in PDA for anti-cancer drug delivery.

Qin et al.[126] fabricated PDA liposomes for "remote controlled" drug delivery that instantaneously release encapsulated reagents upon trigger by a laser. Specifically, partially polymerized liposomes (PPLs) composed of two diacetylenes and a phospholipid were functionalized with gold nanoparticles (GNPs). Notably, the report of laser-induced liposome release via GNPs was previously studied by Wu et al., [127] in which the use of near infrared (NIR) pulse lasers caused release of liposomes due to the raised temperature of the GNPs. First, PPL-GNPs were loaded with fluorescence dye, calcein, to observe the retention ability of the PDA liposomes. After 3 days at 40 °C, negligible leakage was observed. Next, to evaluate the laser-induced release of liposome-encapsulated reagent, PPL-GNPs were loaded with radioactive Tc-99m mebrofenin. Upon irradiation of 10 x 6 ns pulses of laser, PPL-GNPs released approximately 70% of their contents. Finally, PPL-GNPs were loaded with anti-cancer drug, doxorubicin and exposed to breast cancer cells *in vitro*. After laser irradiation, only ~20% of cancer cells were

viable. Significantly, control studies with treatment of cancer cells with unloaded PPL-GNPs determined that laser treatment did not affect the viability of the cancer cells, indicating that the observed drop in cell viability was indeed a result of doxorubicin release from PPL-GNPs.

While other *in vitro* studies of PDA drug delivery liposomes have been reported,[120, 128, 129] Mackiewicz et al.[130] recently reported an *in vivo* study on the use of PDA micelles for both imaging and drug delivery. In particular, PCDA monomers were functionalized with either nitrilotriacetic acids (NTA) or poly(ethylene glycol) (PEG). Specifically, two types of PDA-PEG micelles were investigated, one that was 350 Da (PDA-PEG350) and another that was 2000 Da (PDA-PEG2000). Characterization by DLS indicated that the average diameters of micelles were 7.8 nm and 12.6 nm, respectively. The PDA micelles were further conjugated with fluorescent probes for tracking *in vivo*. NIR imaging of PDA micelles indicated that 2.7 ± 1.9% of injected PDA-PEG2000 micelles were uptaken by the breast cancer xenografts in mice, which was the highest of the three investigated micelle types. A similar study by Yao et al.[131] demonstrates that peptide functionalized PDA micelles loaded with hydrophobic cancer drug camptothecin (CPT), can also be used for targeted drug delivery. DLS and TEM confirmed that the PDA-CPT liposomes were approximately 27 nm in diameter. Sub-30 nm PDA-CPT micelles penetrate tumor vasculature and increase accumulation of the drug, thereby increasing the therapeutic efficacy. Intravenous injections of PDA-CPT caused an order of magnitude increase in therapeutic effect compared to CPT alone in mouse xenograft models of ovarian cancer. Next, to investigate the viability of PDA micelles for the delivery of hydrophobic imaging agents, PDA-PEG2000 micelles were loaded with NIR lipophilic carbocyanine dye (DiR). NIR imaging confirmed that PDA-PEG2000-encapsulated DiR accumulated at the tumor site, whereas non-encapsulated DiR accumulated in the liver. Finally, the anti-cancer agent, paclitaxel (PTX) was loaded into PDA-PEG2000 micelles and studied for their drug delivery capabilities. After 2 months, tumors treated with PTX-loaded PDA micelles showed reductions in volume by a factor of 4.5. As a comparison, tumors treated with commercially available Taxol exhibited reductions in volume by a factor of 3.3. Significantly, it was also reported that PTX-loaded PDA micelles were stable at 4 °C after storage for two months and PTX retained its cytotoxicity within micelles.

While the current use of PDA materials in drug delivery systems does not take advantage of its putative chromatic

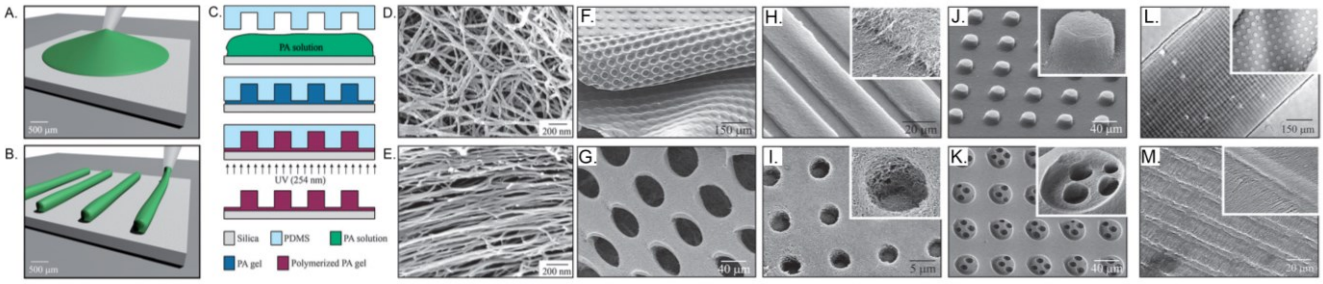


Figure 14. Micropatterned scaffolds for tissue engineering using peptide-conjugated PDA. Various techniques for scaffold fabrication (A-C) create uniform or randomly oriented nanofibers (D, E), removable layers (F), holes (G), microtextures (H), pores, posts (I), (J), two-level topographies (K), and channels (L,M). This method produces PDA scaffold features down to 5 μm in size. Adapted from ref. [132] Copyright 2009, *Royal Society of Chemistry*.

properties, it is important to observe materials beyond their most apparent properties in order to fully take advantage of their capabilities. For example, traditional PDA sensors have all been designed for *in vitro* applications. However, such investigations into PDA materials for drug delivery systems have highlighted their non-toxic, biocompatible properties which give rise to another potential for the design of *in vivo* PDA sensors similar to one we have previously proposed.[133]

5.3 Tissue Engineering

Over the last decade, there has been an increasing interest in the use of peptide amphiphiles in the field of regenerative medicine for the fabrication of tissue scaffolds.[134-136] While synthetic polymeric hydrogels have long been the focus of tissue engineering applications, their limits in the control of pore size, fiber diameter, and shape have posed significant challenges in the field.[137] Alternative approaches, such as electrospinning and phase separation are again limited by lack of morphological controls at the molecular level.[34] Peptide amphiphiles offer significant advantages in their potential to self-assemble into supramolecular structures, including one-dimensional monolayers,[138] two-dimensional β-sheets,[139] and three-dimensional nanotube fibers.[140, 141] However, one of the major challenges in this approach is the poor mechanical stability of unpolymerized peptide amphiphiles.[138] Additionally, chemically cross-linking materials often involves the use of toxic crosslinking agents, resulting in unwanted by-products.[135]

Accordingly, investigations into photopolymerized peptide-diacetylenes have advanced the potential for peptide amphiphiles in tissue engineering applications. Particularly, the photo-inducible polymerization of PDA offers an avenue to fabricate tissue scaffolds with supramolecular structures that have increased mechanical stability without the un-wanted byproducts that often result from chemical cross-linking. While the potential for PDA materials for biosensing applications has been extensively reviewed, their recent applications in tissue scaffolds remains largely obscure. In this subsection, we review a number of recent reports exploring the use of PDA-peptides in tissue scaffolds for tissue engineering.

The first report of PDA in tissue engineering was published in 2006 by Tirrell and colleagues, in which a TCDA-fatty acid monolayer exhibiting varying concentrations of conjugated cell-adhesion peptides fabricated on an LB film was investigated.[138] The cell-adhesion peptide used in this study was glycine-arginine-glycine-aspartic acid-serine-proline (GRGDSP), a peptide sequence well known for its mouse fibroblast adhesion properties. AFM inspection of the composite TCDA-fatty acid and TCDA-GRGDSP at varying molar concentrations of the latter confirmed the adjustability of monomer packing. Further investigation of the TCDA-fatty acid/GRGDSP film studied the adhesion potential of mouse fibroblasts. Specifically, mixed films with TCDA-GRGDSP at molar fractions from 0% to 75% were compared. Cell adhesion assays were performed by treating PDA surfaces with a defined number of cells for 60 minutes and counting the number of cell attached per surface area. The resulting analysis of these assays indicated that films composed of 10% TCDA-GRGDSP exhibited the highest percentage of adherent cells. Significantly, optical micrographs of PDA films after initial cell seeding, mechanical removal of cells, and re-seeding of cells confirmed that PDA films were viable for multiple re-seeding of cells.

This initial two-dimensional study by Tirrell and colleagues was a significant demonstration of the viability of PDA materials for cell adhesion and was further investigated by Frauenrath and colleagues in the formation of three-dimensional scaffolds composed of oligopeptide-functionalized PDA that formed β-sheets in solution.[139] In this communication, TEM and SEM images of their functional PDA confirmed self-assembly into controlled helical fibrils composed of β-sheet aggregates. Importantly, the formation of β-sheets instead of liposomes enabled fibrous morphologies appropriate for the composition of tissue scaffolds.

The realization of PDA scaffolds for tissue engineering has been pioneered by Stupp and colleagues.[132] In their report, top-down lithography and peptide-conjugated PCDA were combined to fabricate an array of tissue scaffolds with highly controlled micropatterning for the study of human mesenchymal stem cells (hMSCs). Specifically, the study takes advantage of the photopolymerizable properties of PDA

materials to create rigid scaffolds with high mechanical integrity. Stupp and colleagues demonstrated the ability to fabricate microtextures including channels, pores, posts, and double-layer topographies to a resolution of 5 μm (Figure 14). They similarly adopted mixed PDA composed of matrix and peptide-conjugated PDA for cell adhesion. The adhesion peptide used in this study was arginine-glycine-aspartic acid-serine (RGDS) and it was determined that scaffolds consisting of 80% PDA-RGDS were optimal for adhesion of hMSCs. From their investigations, Stupp and colleagues were able to distinguish PDA morphologies that were optimal for hMSCs aggregation and differentiation. This significant study demonstrated the great potential for the fabrication of tissue scaffolds with highly controlled micropatterning textures using PDA materials.

Voelcker and colleagues most recently fabricated lysine-conjugated PDA tissue scaffolds for hMSCs.[34] In this report, researchers demonstrated the ability to change the hydrophobic and hydrophilic properties of the lysine-PDA monomers by masking and un-masking lysine side chains using 9-fluorenylmethoxycarbony (Fmoc) and t-butyloxycarbonyl (Boc) protecting groups. This aspect of tissue scaffold production is significant in controlling its wettability, which is an important factor for cell adhesion. Specifically, five polymers with increasing hydrophilicity were investigated. Notably, cell adhesion was most efficient on hydrophilic polymers (contact angles < 32º), but cell proliferation was more favorable on the more hydrophobic polymers (contact angles > 84º).

The pioneering field of PDA materials in tissue engineering is one in which much more investigation is needed. The most recent advances by Stupp and Voelcker demonstrate great potential for PDA tissue scaffolds; however, further explorations might take advantage of the chromatic properties of PDA materials in determining cell properties, such as the detection of cell signals that indicate stem cell differentiation. Additionally, future investigations in the fabrication of biodegradable PDA scaffolds, through mixed amphiphile/PDA composites or side chain modifications, will also be advantageous for the field of bioimplantable materials.

6. FUTURE DIRECTIONS FOR PDA MATERIALS

Since their first preparation nearly a half-century ago, PDA materials have attracted much attention from researchers for their remarkable optical properties. In this article, we have discussed the basic properties of PDA materials and the currently accepted theory behind their environmentally induced color transitions and fluorogenic "turn-on" characteristic. For the benefit of researchers from all fields, we have reviewed some general procedures in PDA synthesis and modification and highlighted more recent explorations. These include PDA-epoxy intermediates and the use of modified functional groups (e.g. -OH, $-NH_2$, -COOH) on matrix constituents to prevent non-specific binding of biomolecules. We have also discussed the most common characterization methods for PDA and compared the traditional approach to quantify color transitions (CR), with a new, simpler method developed by Kolusheva, Jelinek, and colleagues (DCA). Finally, we have reviewed advancing platforms for PDA materials, including paper-based PDA sensors, PDA systems for drug delivery, and PDA nanostructures for tissue engineering.

As PDA technology advances and continues to branch into diverse applications, it is critical to investigate and understand the underlying mechanisms behind its chromatic properties. While the development of new sensor types and novel applications are exciting aspects to the field of PDA materials, its full potential cannot be realized without a fundamental understanding of its properties.

Future explorations of PDA applications may combine its "conventional" appeal as biosensors with the more recent investigations in biological and biomedical applications. Even more, with the recent developments of reversible PDAs (not discussed in this article[62, 65, 66, 142], advancements in the previously suggested field of *in vivo* sensors has great potential in the fields of medicine, horticulture and agriculture to monitor, diagnose and even treat diseases in humans, animals, and plants alike. Alternatively, the advancement of low-cost and stable paper-based PDA sensors are ideal for low-resource applications and may be coupled with smartphone-based systems for quantitative analyses.

As the field of PDA materials garners more attention in the scientific world, PDA applications will continue to expand and increase in quality and sensitivity. Already, many reports demonstrate the fabrication of materials comparable to, or even better than, currently accepted standards. Eventually, with the current rate of developments, practical application of PDA systems will be available and accessible to both research and commercial sectors across the globe.

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