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Analysis

Chromatic polymer assays for the analysis of lipid and lipoprotein peroxidation

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Summary

Evaluation of the extent of lipoprotein oxidation and the effects of therapeutic and dietary agents upon lipid peroxidation processes are critical in understanding and combating atherosclerotic diseases. Bioanalytical chromatic assays based upon polydiacetylene (PDA) have recently emerged as a useful platform for analysis of lipoprotein oxidation. This article describes the principles of PDA-based sensing, and its application to the study of lipids peroxidation, both in purified lipoprotein samples, as well as in blood plasma specimens.

Introduction

Oxidative stress is considered a major risk factor for the development of atherosclerosis. Lipid peroxidation in plasma, lipoproteins, and arterial cells is intimately related to several pathological conditions associated with atherosclerosis development, such as hypercholesterolemia, hypertriglyceridemia, diabetes, and renal failure. Macrophage accumulation of oxidized lipids is the hallmark of early atherogenesis, and it is the result of cellular uptake of oxidized low density lipoproteins (Ox-LDL), as well as cellular lipids peroxidation. Up to now there has not been developed a reliable methodology to measure the type and the extents of lipid peroxidation, as this process is very heterogenous in nature, involving various reactive oxygen species (ROS) and reactive nitrogen species (RNS). Furthermore, the extent of plasma and that of lipoproteins oxidation is considerably affected by endogenous and exogenous antioxidants in the plasma. This article summarizes our recent studies aimed at developing novel colorimetric platforms for investigating lipid peroxidation, both *in vitro* and *in vivo*.

Polydiacetylene biosensors

Polydiacetylene (PDA) was first synthesized in the late 1960s [1, 2] and has captured the imagination of scientists and technologists alike due to its unique chromatic properties. Specifically, diacetylene monomers can be aligned in solutions and polymerized through ultraviolet (UV) irradiation, producing a conjugated PDA network [3, 4] (**Figure 1**). The unique feature of PDA systems is their absorbance of light in the visible spectral region, thereby exhibiting colour, in most cases blue [5, 6]. Moreover, conjugated PDA can undergo dramatic colorimetric transformations that are visible to the naked eye, induced by varied soluble analytes and environmental perturbations (Figure 1). Another attractive feature of PDA systems in the context of sensing applications has been the fluorescence properties; blue phase PDA is non-fluorescence while the red-phase configuration exhibits high fluorescence with minimal bleaching [7–9]. The chromatic (color/fluorescence) properties of PDA have inspired numerous *sensing* applications. In particular, the incorporation of diverse molecular recognition elements within PDA assemblies has made PDA a highly versatile sensing platform. Importantly, since the PDA headgroups are intrinsically negatively-charged [10], many reported biosensing applications have focused on detection of positively-charged amphiphilic molecules [11]. Notably, biological sensing and analysis platforms were also demonstrated that were based on *mixed lipid/PDA* assemblies (vesicles, films, sol-gels, others). The lipid molecules in such composite systems often adopt *bilayer* organizations, thereby mimicking membrane environments and constitute targets for membrane-active and/or amphiphilic substances [12, 13].

Colorimetric analysis of lipoproteins using PDA-based vesicles

Biomimetic lipid/PDA vesicles have been successfully employed for analysis of lipoprotein oxidation through monitoring their bilayer interactions. Lipoproteins are membrane active since their inherent physiological roles are intimately linked to targeting and capturing lipophilic molecules, particularly cholesterol [14, 15]. Lipoproteins are also known to dock onto membrane-embedded receptors, such as LDL binding to the LDL receptor [16]. **Figure 2** depicts a representative colorimetric analysis of low density lipoprotein (LDL) and high density lipoprotein (HDL) using lipid/PDA vesicles. As described above, the lipid constituents within the mixed lipid/PDA vesicles mimic membrane bilayers and as such constitute a target for lipoprotein interactions. Accordingly, the lipid/PDA vesicles platform represents a simple color assay designed to probe the extent of membrane binding of the lipoproteins, Figure 2A presents a scanned image of a multi-well plate containing dimyristoylphosphatydilcholine (DMPC)/PDA vesicles (mole ratio 2 : 3) reacting with different HDL samples (similar results, qualitatively, were recorded in case of LDL, see Figure 2B). Notably, the vesicles exhibited distinct color responses that were clearly dependent upon the oxidation state: while HDL isolated from blood plasma of healthy individuals gave rise to pronounced blue-purple/red

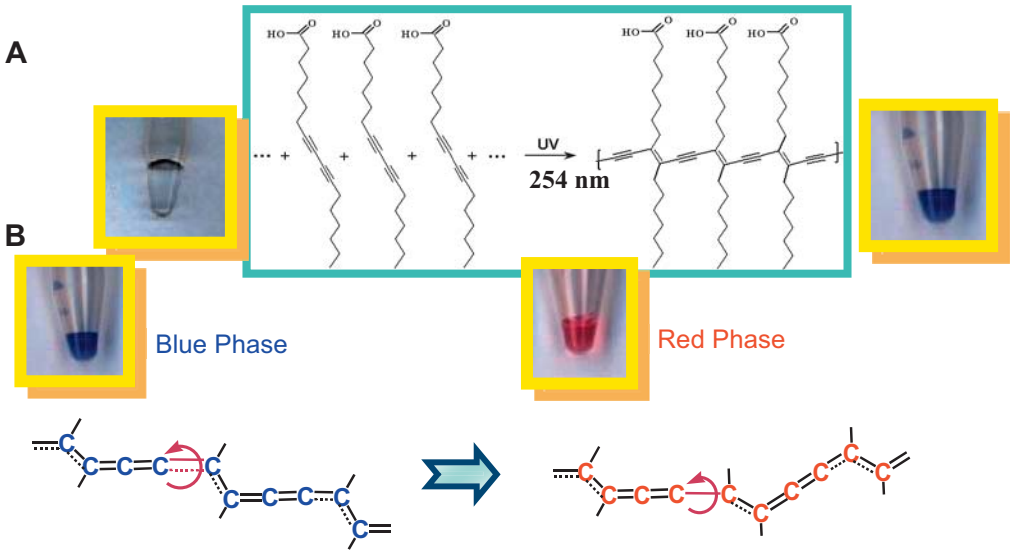

Figure 1. Color properties of polydiacetylenes. (A) UV-induced polymerization of diacetylene monomers yielding the blue polydiacetylene (PDA) network; (B) Blue-red transitions of PDA associated with disruption of the conjugated system.

transition (Figure 2A,ii), HDL obtained from diabetic patients (Figure 2A,iii) or HDL oxidized *in vitro* (Figure 2A,iv) induced lesser color transformations. These results reflect significantly more attenuated *bilayer interactions* of oxidized lipoproteins compared to non-oxidized particles.

The bar diagram in Figure 2B provides a *quantitative* depiction of the colorimetric response of both HDL and LDL using two lipid/PDA models comprising different lipid molecules in addition to the PDA matrix – DMPC/PDA (grey bars in Figure 2B) and DMPE/DMPG/PDA (black bars). The results summarized in Figure 2B demonstrate that less pronounced blue-red transitions were apparent for oxidized lipids both in case of the LDL as well as the HDL system. The colorimetric data further show that the colorimetric transformations were dependent upon the lipid constituents within the vesicles, confirming the importance of *lipid interactions* of the tested lipoproteins in affecting the color changes. Indeed, the significantly different color transformations induced by HDL and LDL (Figure 2B) are likely ascribed to the effect of *particle density* in vesicle interactions and consequent colorimetric transformations. It should be noted that while the precise mechanistic aspects pertaining to the distinct colorimetric response associated with lipoprotein oxidation have not yet fully deciphered, the data in Figure 2

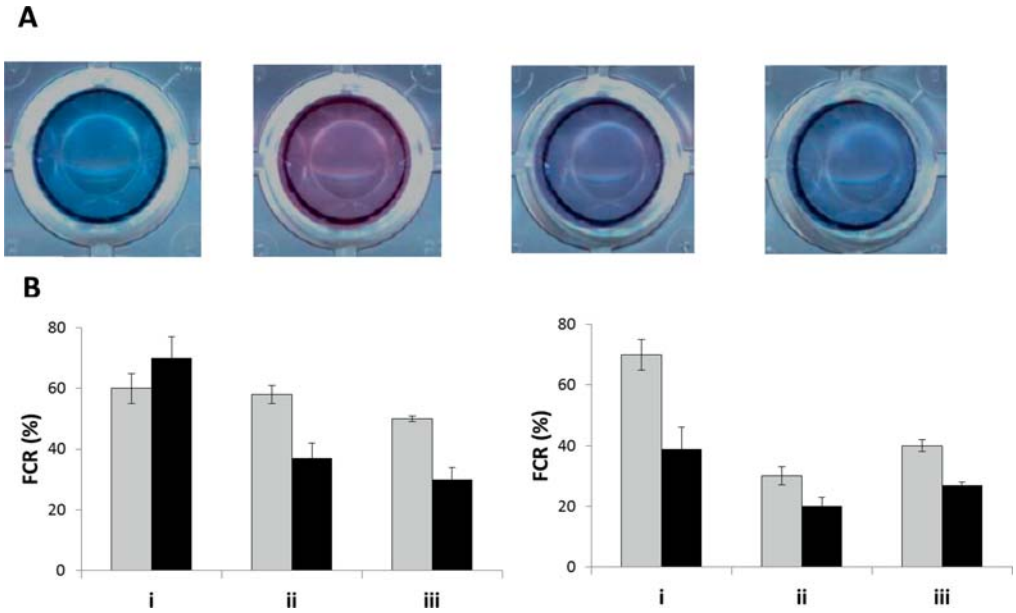

Figure 2. Color transitions of lipid/PDA vesicles induced by lipoproteins. (A) Color changes induced by low-density lipoprotein (LDL), from left: control vesicles (no lipoprotein added); LDL added; LDL extracted from diabetic patients; LDL oxidized *in vitro*. (B) Quantitative colorimetric analysis. Left diagram: LDL; Right diagram: HDL. In both cases the grey bars were recorded for 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/PDA vesicles (2 : 3 mole ratio) and black bars for 1,2-dimyristoyl-sn-glycero-3-phospho-ethanolamine (DMPE)/1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG)/PDA vesicles (1 : 1 : 3 mole ratio).

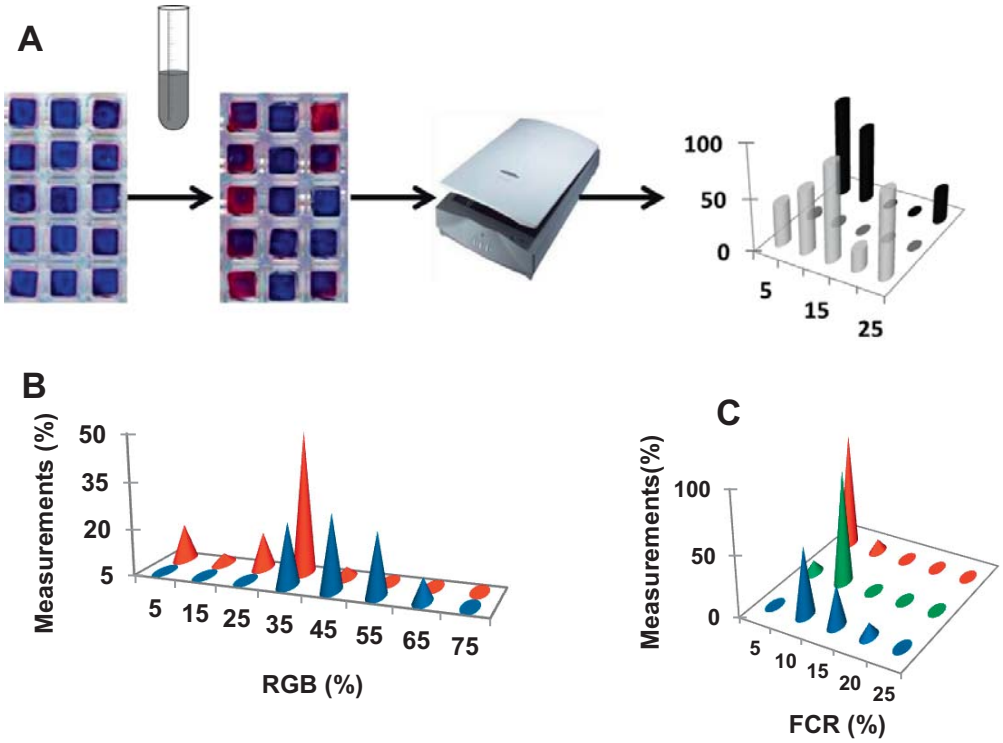

Figure 3. Colorimetric screening of blood plasma using lipid/PDA/sol-gel. (A) Schematic description; (B) comparison between blood plasma obtained from healthy persons (blue) or hypercholetrolemic patients (red); (C) comparison of plasma color responses from healthy persons (blue), hypercholetrolemic patients (red), or healthy plasma that was oxidized *in vitro* (green).

demonstrate that the lipid/PDA vesicle system could provide a simple visual means for distinguishing among lipoprotein species and assessing lipoprotein oxidation.

Color screening of lipid peroxidation in blood plasma

While the representative color data in Figure 2 were recorded *in vitro* – e.g. lipoprotein samples extracted and purified from blood plasma, the PDA technology can be also implemented for screening of *whole plasma* and potentially employed as a diagnostic platform. **Figure 3** depicts a novel approach in which the colorimetric sensitivity of PDA to lipid oxidation was employed as a vehicle for hypercholesterolemia (increased blood cholesterol levels) diagnosis. Instead of trying to identify specific biomarkers in plasma such as lipid concentration profiles, the diagnosis methodology exploited interactions between *whole plasma* samples and lipid/polydiacetylene (PDA) vesicles embedded within a transparent sol–gel matrix.

Figure 3A illustrates the diagnostic procedure. The colorimetric platform in a multi-well plate format is prepared through embedding the lipid/PDA liposomal nanoparticles within a transparent silica-based sol-gel matrix, designed to endow high stability to the sensor system [17]. Blood plasma samples were then added to the lipid/PDA/sol-gel compartments, giving rise to visible blue-red color transformations. The entire plate is then placed upon a conventional desktop scanner and the color images are analyzed through a simple red-green-blue (RGB) analysis algorithm, generating a quantitative measure for the plasma-induced color transitions.

The diagram in Figure 3B presents the colorimetric data obtained through application of the diagnostic PDA assay on blood plasma. Essentially, the x axis corresponds to a specific *range* of colorimetric transitions (i.e. "5" corresponds to % RGB values between 0–10; "15" corresponds to %RGB values between 10–20, etc. The maximal blue-red transition is defined as % RGB of 100) [18]. The numbering on the Y axis indicates the percentage of measurements (plasma samples and repetitions) recorded in each % RGB range. The colorimetric data shown in Figure 3B indicates that the PDA assay can distinguish between plasma samples obtained from healthy and hypercholesterolemic patients. Specifically, Figure 3B shows that the plasma samples obtained from *healthy* individuals (distribution of recorded %RGB transitions shown in *blue* in Figure 3B) overall induced *more pronounced* blue-red transformations as compared to plasma samples obtained from hypercholesterolemic patients (*red* area in Figure 3B). For example, the blue bars in Figure 3B indicate that most measurements of plasmas from healthy persons induced % RGB of between 40% and 60% while the corresponding values for the hypercholesterolemic plasma samples were between 20% and 30% (red bars).

To account for the colorimetric differences apparent in Figure 3B, a colorimetric analysis was carried out using lipid/PDA vesicles incubated with plasma samples from healthy individuals, hypercholesterolemic patients, and healthy patient plasma that were *intentionally oxidized* (Figure 3C). Indeed, Figure 3C shows that plasma samples from healthy persons which underwent chemical oxidation (through incubation with copper sulfate) induced colorimetric response that was significantly *closer* to those induced by plasma from *hypercholesterolemic* patients (green area in Figure 3C). This result indicates that the distinct color response of the lipid/PDA platform upon incubation with plasma obtained from hypercholesterolemic patients is indeed related to lipid oxidation.

Conclusions

PDA systems constitute a useful platform for sensing and analyzing of diverse analytes. In the context of lipoprotein lipids peroxidation in particular, PDA assemblies might be advantageous since the lipid/polymer systems essentially mimic biological membranes and as such exhibit high sensitivity to even slight variations in membrane interactions of target analytes. Indeed, lipid peroxidation is expected to alter the physico-chemical properties of lipoprotein particles, thereby modulating their amphiphilic properties and/or membrane interactions which is translated into distinct colorimetric response of the PDA sensor.

The wide body of published reports on the use of PDA in biosensing and diagnostic applications in particular suggest that this technology could be implemented as a viable practical tool for lipid peroxidation screening and investigations. In particular, PDA systems offer notable practical benefits, including visible sensing capabilities (through easily discerned color changes), inexpensive chemical building blocks and simple preparation procedures, long term stability of the sensor, and varied sensor configurations (soluble vesicles, sol-gel matrixes, and thin films). Overall, PDA-based assays will likely continue to contribute to analysis and biomedical applications of oxidized lipoproteins.

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