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ORIGINAL RESEARCH ARTICLE

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Abstract
Objective Hypercholesterolemia (increased blood cholesterol level) is considered a major risk factor for developing atherosclerotic diseases. As such, alerting individuals on hypercholesterolemic conditions is a crucial component in averting onset of atherosclerosis and its outcome—cardiovascular diseases. While common diagnostic tools such as cholesterol and lipoproteins determination are widely employed for hypercholesterolemia screening, their effectiveness has been questioned since they do not shed light on critical physiological factors like lipid oxidation and inflammation levels, which constitute prominent determinants for development of atherosclerotic diseases. The objective of this study is to develop a simple assay for identifying hypercholesterolemia, and assessing the impact of therapeutic treatments.
Methods We developed a diagnostic assay based upon color transformations of polydiacetylene, a unique conjugated polymer, upon interactions with blood plasma obtained from healthy individuals, hypercholesterolemic patients, hypercholesterolemic patients treated with statin, and hypercholesterolemic patients treated with statin together with pomegranate extracts. The color transformations of the polymer were monitored through desktop color scanning combined with colorimetric image analysis.
Results We show that the colorimetric assay was able to distinguish among plasma. Bio-analytical characterization reveals that the distinct colorimetric responses likely arise from interactions with plasma lipoproteins. Importantly, the colorimetric changes are not simply correlated with the relative abundance of cholesterol (or other lipids) in the plasma of hypercholesterolemic or healthy patients, but also reflect the presence of oxidized and inflamed species.
Conclusions This paper introduces a simple color assay for detection of hypercholesterolemia and monitoring the effect of therapies directed at mitigating this physiological condition. The colorimetric system might constitute a novel platform for assessing patient vulnerability towards the development of atherosclerosis.

Key Points

Colorimetric assay to monitor hypercholesterolemia.

Assay sensitive to plasma lipid quality not only quantity.

Application of the assay indicates that statin/pomegranate juice therapies did not revert plasma condition back to the healthy state.

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E. Mann · S. Kolusheva · R. Jelinek (✉)
Faculty of Natural Sciences, The Ilse Katz Institute, Ben Gurion University of the Negev, 84105 Beer Sheva, Israel
e-mail: razj@bgu.ac.il

R. Yossef · A. Porgador
The Shraga Segal Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel

M. Aviram
The Lipid Research Laboratory, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Rambam Health Care Campus, Haifa, Israel

1 Introduction

Hypercholesterolemic patients are characterized by high levels of cholesterol and by oxidative stress and are prone to accelerated atherosclerosis [1–4]. Therapies aimed at addressing hypercholesterolemia are generally focused on lowering cholesterol levels and preventing oxidative stress [5]. Statin therapy is the most common approach for hypercholesterolemia [6, 7]. Recently, the health benefits of pomegranate juice (PJ) have been demonstrated, particularly in relation to hypercholesterolemic conditions. Specifically, it has been shown that consumption of PJ by healthy volunteers significantly decreased their low density lipoprotein (LDL) and high density lipoprotein (HDL) oxidation [8]. Recently, we observed that the combination of pomegranate extract with statin therapy decreased the atherogenicity of serum in hypercholesterolemic patients [9].

Early identification of hypercholesterolemia is a requisite for initiating therapy. The most widely used current diagnosis approach is the routine blood test for measuring the concentrations of fats, including cholesterol, LDL, HDL, and other lipid components. Such blood screening offers advantages like low costs, broad availability, and a relatively short waiting time. However, the main limitation of blood fat determination is the fact that this test focuses on a rather narrow aspect of hypercholesterolemia—concentrations of fat molecules, thereby ignoring the significance of oxidative stress and other potential contributions to onset of atherosclerotic conditions.

We present here a novel approach to hypercholesterolemia diagnosis. Instead of trying to identify specific biomarkers in plasma such as lipid concentration profiles, the diagnosis methodology we developed is based upon reactions between plasma and an artificial biomimetic assay comprising lipid/polydiacetylene (PDA) vesicles embedded within a transparent sol–gel matrix. PDA is a conjugated polymer, which exhibits unique color and fluorescence properties [10]. The initial phase of the polymer is intensely blue and visible to the naked eye, while dramatic color transformations (accompanied by fluorescence emission [10]) are induced by external stimuli—particularly interactions with soluble amphiphilic or membrane-active molecules [11, 12]. In the context of plasma–membrane interactions, the chromatic signals induced by amphiphilic components within plasma constitute the fundamental means for distinguishing between normal and disease conditions [13, 14].

2 Materials and Methods

2.1 Materials

The lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and trimyristin (TM, 1,2,3-trimyristoyl-glycerol) were purchased from Avanti Polar Lipids (Alabaster, AL). The diacetylenic monomer 10,12-tricosadiynoic acid was purchased from Alfa Aesar (Karlsruhe, Germany). Tris(hydroxymethyl)aminomethane (TRIZMA base buffer), and tetramethoxysilane (TMOS) were purchased from Sigma-Aldrich. Pomegranate extract pill (POMx) was purchased from PomWonderful LLC, Los Angeles, CA. POMx was stored in vegan capsules, and each capsule contains 1,000 mg of natural pomegranate polyphenol extract, which constitutes 650 mg of gallic acid equivalents, which is equal, in polyphenol content, to one glass (8 ounces) pomegranate juice. The vegan placebo pills were matched by size and shape to the POMx pills and were kept in similar brown bottles at room temperature. Each placebo pill contained 520 mg lactose monohydrate. Placebo pills were provided by the pharmacy service at Rambam Health Care Campus.

2.2 Patients

Fourteen healthy male adults with statin-naïve hypercholesterolemia and 10 hypercholesterolemic male adults were enrolled in the study. All the subjects were otherwise healthy, and were known from regular follow-up in primary care clinics. They all signed an informed consent prior to any study-related procedures. The clinical and demographic features of all patients were registered. The healthy male adults with statin-naïve hypercholesterolemia were randomly assigned to one of two groups; in the first group ($n = 11$), the patients were treated by simvastatin (20 mg/day) and 1 POMx pill/day and in the second group ($n = 3$), the patients were treated by simvastatin (20 mg/day) and a vegan placebo pill. Randomization was performed with the use of sealed, opaque, serially numbered envelopes, each of which contained a box with 30 tablets of simvastatin 20 mg, together with a bottle containing 30 capsules of either POMx or matching placebo vegan capsules. Unblinding was performed only at the end of the treatment period. Fasting blood samples were drawn in the morning from all patients at baseline and after one and two months of treatment. Monocytes were isolated from the blood of all patients in each group, at baseline and after 2 months of treatment, and also from healthy and hypercholesterolemic subjects. The study was approved by the local ethics committee of Rambam Health Care Campus, Haifa, Israel (approval number 0353/12).

2.3 Lipid/PDA/Sol–Gel Matrix

Chromatic vesicles comprising the diacetylene monomer 10,12-tricosadiynoic acid, TM and DMPC (3:0.5:1.5) were dissolved in chloroform/ethanol (1:1) and dried together

in vacuo to constant weight, followed by addition of double-ionized water to a final concentration of 7 mM and probe-sonicated at 70 °C for 10 min. The vesicle solution was subsequently cooled at room temperature, diluted with Tris buffer pH 7.5 (1:1), and used for sol–gel preparation. Silica sol–gel preparation was based upon an alcohol-free method [15]. Briefly, a mixture of all gel constituents was prepared [TMOS:water:HCl (0.62 M) at 4.41:2.16:0.06 volume ratio]. The mixture was stirred vigorously for 1 h at 4 °C to obtain a homogeneous solution. To remove the alcohol, the solution was further diluted with water 1:1 and evaporated by a rotor evaporator (Buchi, Germany) for approximately 6 min at a pressure of 60 mbar. The lipid/diacetylene vesicles in buffer were then added to the silica solution (volume ratio of 1:1) and the suspension was immediately placed in the wells of 384-well ELISA plates (Grainer, flat bottom). Each well contained 15 μL silica/liposomes mixture solutions. Gelation was carried out for 30 min at room temperature followed by addition of Tris pH 7.5 for long-term storage in the refrigerator. Polymerization of the diacetylene monomers to produce the blue-phase TM/DMPC/polydiacetylene (PDA)/gel construct was carried out after overnight refrigeration using 2-min irradiation at 254 nm in a 80 W Cross-Linker BLX-254 (Vilber Lourmat, France).

2.4 Gel-Chromatic Analysis

Aliquots of the tested solutions were placed in the multiwell plate and incubated at 37 °C for 3 h. During incubation, the multiwell plates were scanned in transmitted mode on an Epson 4990 Photo scanner every 15 min to produce 2,400 dpi, 24-bit color depth red–green–blue (RGB) images. Digital colorimetric analysis (DCA) was carried out by extracting RGB channels values for each pixel within the sample spots in the scanned images, and the color change values were calculated using Matlab R2010 scientific software (The Mathworks, Inc., MA, USA) as detailed previously [16]. Briefly, DCA utilizes the standard "red–green–blue" (sRGB) model, essentially translating every color signal into three distinct values corresponding to the intensities of red (R), green (G), and blue (B) color channels [17]. Accordingly, the relative intensity of a particular RGB component in a scanned image can be defined as the chromaticity level. For example, the red chromaticity level (r) in each pixel was calculated as

$$r = \frac{R}{R + B + G} \tag{1}$$

where R (red), G (green), and B (blue) are the three primary color components. For a defined surface area within a PDA-based sensor well, we classify a quantitative parameter denoted chromatic response that represents the total blue–red transformations of the pixels encompassed in the area,

$$\%RGB = \left(\frac{r_{\text{sample}} - r_0}{r_{\max} - r_0}\right) \times 100\,\% \tag{2}$$

where r_{sample} is the average red chromaticity level of all pixels in the scanned surface, r_0 is the average red level calculated in a blank surface (blue sensor well, which only buffer was added to), and $r_{\max}$ is the average red chromaticity level of the maximal blue-red transition, an area of the sensor well in which the most pronounced blue-red transition was induced (positive control, usually achieved by incubation with a strong base such as NaOH 1 M). In essence, the calculated *%RGB* is the normalized change in the red chromaticity level within the sensor well surface on which the tested sample was deposited.

2.5 Size Fractionation

Plasma samples in a volume of 100 μL each were thawed on ice for 1 h. Hundred kDa centricons (YM-100, Millipore™) were washed three times with 100 μL of PBS buffer pH 7.4, and 100 μL thawed plasma samples were loaded and centrifuged for 90 min at 4 °C at 5,000×g. The >100 kDa fraction was collected from the upper part of the centricon and was diluted by PBS buffer pH = 7.4 up to volume of 100 μL. The <100 kDa fraction was separated again to two fractions of <30 kDa and 30–100 kDa by the same process described above using 30 kDa centricons. The fraction solutions were placed in a freezer until experimentation.

2.6 Lipid Oxidation

Healthy plasma samples were thawed on ice for 1 h prior to incubation at 37 °C in air for 24 h using 35 μmol/L of freshly prepared $CuSO_4$. Oxidation was terminated by refrigeration at 4 °C and addition of 1 mM Na_2EDTA.

2.7 Lipid/Polydiacetylene Vesicle Fluorescence Assay

TM/DMPC/PDA vesicles (0.5:1.5:3 mole ratio) were prepared as described above (gel preparation). Samples for fluorescence measurements were done in triplicates and were prepared by adding 2 μL of >100 kDa plasma fraction sample to 30 μL vesicles followed by addition of 30 μL 50 mM Tris base buffer (pH 8.0). Fluorescence was measured on a Fluscan Ascent using a 96-well microplate (Grainer), using excitation of 544 nm and emission of 620 nm using LP filters with normal slits. Fluorescent chromatic responses were calculated according to the formula:

$$\%\mathrm{FCR} = \left(\frac{\mathrm{Em}_i - \mathrm{Em}_0}{\mathrm{Em}_{\mathrm{red}} - \mathrm{Em}_0}\right) \times 100\,\% \quad (3)$$

in which Em_i is the value obtained for the vesicle solution after incubation with the plasma sample, Em_0 is the value obtained for the control well (vesicles treated only with buffer) and $\mathrm{Em}_{\mathrm{red}}$ is the maximal fluorescence value obtained for the red-phase vesicles (vesicles heated at 80 °C for 2 min). The chromatic results reported represent statistical distribution of multiple measurements of >100 kDa plasma fractions from different patients.

2.8 Electron Spin Resonance (ESR) Measurements

The spin probe 4-(decyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT-10) was synthesized according to published protocols (see Supporting Information) and added to the vesicle samples at a molar ratio of 1:50 (CAT-10:DMPC) from ethanol solution. 4 μL plasma samples added to 15 μL vesicle solution were placed in a 20 mm length, 1 mm inner diameter quartz capillary and recorded using an EPR-mini X-band spectrometer (Spin Ltd., Russia) at room temperature. The modulation 10 G, time constant 0.01, field range 3330–3370 G and the microwave power level were chosen at subcritical values 20 mW, to reach the best signal/noise ratio. The following equation was used to determine the rotational correlation time (τ_c):

$$\tau_c = 6.6 \times 10^{-10} \cdot \Delta H_{(+1)} \left(\sqrt{\frac{I_{(+1)}}{I_{(-1)}}} - 1\right) (\mathrm{sec}), \quad (4)$$

in which τ is the rotational correlation time, 6.6×10^{-10} is the constant pertinent to the nitroxide spin label, $\Delta H_{(+1)}$ the low field line width, $I_{(+1)}$ the low field line height and $I_{(-1)}$ the high field line height, respectively.

3 Results and Discussion

Figure 1 illustrates the procedure for application of the diagnostic assay. The colorimetric platform in a multi-well plate format is prepared through embedding liposomal nanoparticles comprising lipids and diacetylene monomers within a transparent silica-based sol–gel matrix [18]. Following irradiation with ultraviolet light, the PDA network is formed, producing the blue appearance of the lipid/PDA/sol–gel (Fig. 1a). Blood plasma samples were then added to the lipid/PDA/sol–gel compartments, giving rise to visible blue–purple and blue-red transformations after 1–2 h incubation (Fig. 1b). Importantly, the differences among the colorimetric transformations induced by the plasma samples are the core feature of the new diagnostic assay. To facilitate quantitative analysis of the colorimetric transformations, the lipid/PDA/gel platform was color-scanned (using a conventional desk-top scanner, Fig. 1c), and the recorded images were analyzed using a simple red–green–blue (*%RGB*) algorithm, generating numerical values reflecting the extent of blue–red transitions (Fig. 1d). [The specific equation for *%RGB* is outlined in the Sect. 2]. Essentially, higher *%RGB* values correspond to more pronounced blue-red transition (i.e. more red appearance).

Figure 2 graphically summarizes the colorimetric changes of trimyristin (TM)/dimyristoylphosphatidylcholine (DMPC)/PDA (0.5:1.5:3 mole ratio) vesicles embedded in a sol–gel matrix, following incubation with plasma samples obtained from four patient groups: healthy persons, hypercholesterolemic patients (as determined by their high plasma LDL cholesterol level of over 130 mg/dL, see Table 1, SI), hypercholesterolemic patients treated for 6 weeks with simvastatin, and hypercholesterolemic patients treated for 6 weeks with simvastatin together with pomegranate juice extract. The bar diagrams in Fig. 2 display the entire distribution of the colorimetric datapoints recorded for all plasma samples. 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). The numbering on the *y* axis indicates the percentage of measurements (plasma samples and repetitions) recorded in each *%RGB* range.

The bar diagrams in Fig. 2 demonstrate statistically distinguished colorimetric groupings induced by the different patient groups (the colorimetric results are presented numerically in Table 2, SI). Specifically, Fig. 2a shows that the plasma samples obtained from healthy individuals (distribution of recorded *%RGB* transitions shown in red in Fig. 2a) overall induced more pronounced blue–red transformations as compared to plasma samples obtained from hypercholesterolemic patients (blue area in Fig. 2a). For example, the red bars in Fig. 2a 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 %. The colorimetric experiment summarized in Fig. 2a is significant, since it demonstrates that the TM/DMPC/PDA/sol–gel diagnostic assay is capable of distinguishing, in a statistically significant manner, between blood plasma of healthy and hypercholesterolemic patients.

Figure 2b displays the bar diagram encompassing the entire colorimetric database, including color transitions induced by plasma samples obtained, respectively, from healthy persons (red area), hypercholesterolemic patients (blue), hypercholesterolemic patients treated with simvastatin (green), and hypercholesterolemic patients treated with simvastatin + pomegranate extract (orange). From a

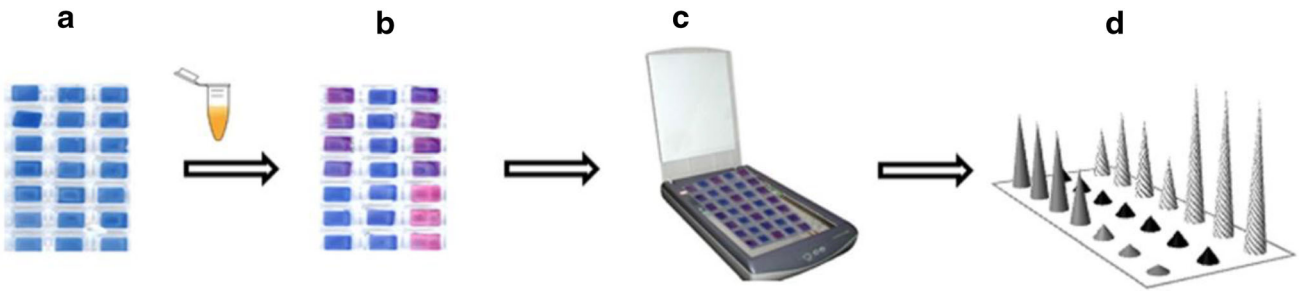

Fig. 1 Experimental scheme. **a** Lipid/polydiacetylene/sol–gel in a multi-well plate format; **b** plasma samples are added to each well, inducing blue–red transformations within some wells depending upon composition; **c** the plate is scanned using a conventional desktop scanner; **d** following application of an image analysis algorithm numerical values are obtained, reflecting the extent of blue–red transitions in each well

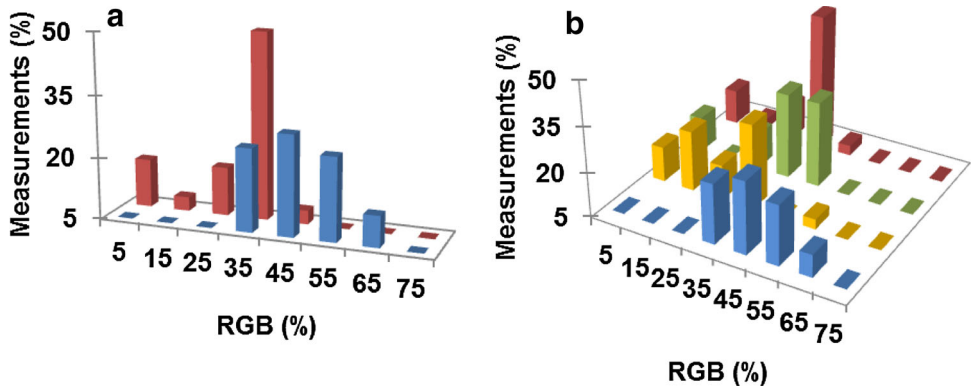

Fig. 2 Colorimetric bar diagrams. Diagrams showing the statistical distribution of color transitions (i.e. *%RGB*) induced in trimyristin/DMPC/PDA/sol–gel upon incubation with plasma samples obtained from all patients. **a** healthy persons (*red*) and hypercholesterolemic patients (*blue*); **b** healthy persons (*red*), hypercholesterolemic patients (*blue*), hypercholesterolemic patients treated with simvastatin (*green*), hypercholesterolemic patients treated with simvastatin + pomegranate extract (*orange*). The *bar diagrams* demonstrate distinct colorimetric-transition "clusters" corresponding to each patient group

practical standpoint, Fig. 2b underscores the possible use of the colorimetric TM/DMPC/PDA/sol–gel assay as a diagnostic tool for screening hypercholesterolemic patients. First, the bar diagram in Fig. 2b clearly shows clusters of color transformations corresponding to the distinct patient groups (i.e. the color transitions induced by plasma samples extracted from each patient group were not random). Second, according to the colorimetric assay the therapeutic treatments clearly did not revert back the colorimetric profile of blood plasma to the healthy conditions (green and orange areas vs. red area in Fig. 2b).

Another notable result concerns the lipid composition employed in the colorimetric analyses depicted in Fig. 2, specifically vesicles containing TM and DMPC associated with PDA. Several other lipid compositions that were tested did not yield similar groups that were distinguished according to the colorimetric transformations ascribed to the different patient groups. This observation is significant, because it indicates that specific interactions between plasma components and the TM/DMPC lipid assembly are responsible for the colorimetric transitions. Indeed, trimyristin has been shown to affect cholesterol levels in hypercholesterolemic patients [19], pointing to physiological relevance of the colorimetric results.

To shed light upon mechanistic aspects pertaining to the color transformations and to corroborate the results obtained through application of the lipid/PDA/sol–gel platform, we tested the plasma samples using a solution-based TM/DMPC/PDA vesicle assay (Figs. 3, 4) [12], and further applied another biophysical technique designed to illuminate plasma-vesicle interactions (Fig. 5). First, to assess which species in the blood plasma contributes most to the colorimetric transformations, we size-separated plasma samples through centrifugation/filtration and then incubated each fraction with TM/DMPC/PDA vesicles (Fig. 3). The scanned image of TM/DMPC/PDA vesicle solutions in Fig. 3 shows that the most pronounced red lipid/PDA vesicles occurred upon incubation with the plasma fraction containing the largest species (>100 kDa). This observation indicates that particles such as lipoproteins are likely the main plasma components responsible for the colorimetric transformations [14]. Some blue-red

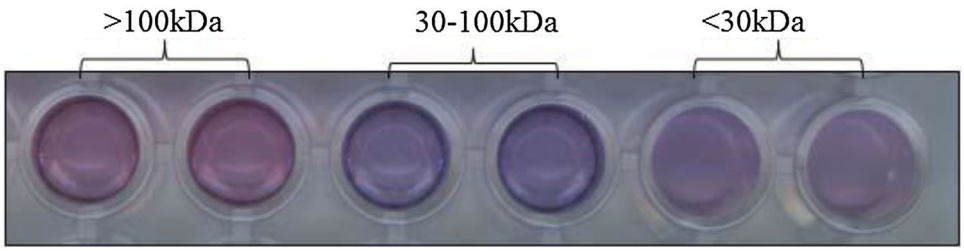

Fig. 3 Color transitions induced by size-separated fractions of plasma samples. Two plasma samples obtained from healthy persons size-separated by filtration and incubated with TM/DMPC/PDA vesicle solutions. Most pronounced blue–red transition observed following addition of the biggest (>100 kDa) plasma particles

change is also apparent in the <30 kDa fraction (Fig. 3, right wells), likely induced by cationic species such as Na^+, K^+, and Ca^{2+}, known to affect color transitions in PDA vesicles [20]. A chromatic experiment examining color transitions induced in TM/DMPC/PDA vesicles by physiological solution (0.9 % NaCl) confirmed that cations such as Na^+ induce blue–red transformations in the vesicles (Fig. 1, Supporting Information).

Figure 4 presents the results of chromatic experiments in which the high-molecular weight fractions of the plasma specimens (>100 kDa, denoted "high MW" plasma samples) were added to the TM/DMPC/PDA vesicle solutions (the colorimetric results are presented numerically in Table 3, SI). The experiments in Fig. 4 depict the percentage fluorescence chromatic response (%FCR) of the vesicles, which is a commonly used measure for the blue-red transformations [10] (i.e. high %FCR corresponds to more pronounced blue-red change). The distribution of data-points from all high-MW plasma samples of the four patient groups in Fig. 4a corroborates the sol–gel results (Fig. 2). In particular, similar to the gel matrix, the vesicle assay reveals distinct colorimetric "clusters" corresponding to each patient condition (the different colored areas in Fig. 4a). Echoing the colorimetric gel analysis of plasma in Fig. 2b, samples obtained from healthy patients overall induced more pronounced blue-red transitions when incubated with the TM/DMPC/PDA vesicles, as compared to hypercholesterolemic plasma samples (Fig. 4a). Also consistent with the sol–gel colorimetric assay (Fig. 2b), the vesicle assay data in Fig. 4a strongly suggest that chemical therapy (within the 6-week time period examined), did not result in complete reversal of hypercholesterolemia-induced changes to blood chemistry.

The colorimetric and fluorescence results in Figs. 2, 3 and 4 clearly show that monitoring lipid profile (i.e. concentration of cholesterol species) is not the sole factor in determining hypercholesterolemia conditions and therapeutic effects. Indeed, lipid oxidation is also believed to have major adverse health implications in conjunction with hypercholesterolemia [21]. To examine this physiological aspect and its relationship with lipid/PDA-based diagnostics we evaluated the colorimetric transformations induced by plasma samples that were intentionally oxidized (Fig. 4b). Strikingly, Fig. 4b shows that high-MW fractions of plasma samples from healthy persons which underwent chemical oxidation (through incubation with copper sulfate) induced fluorescence emissions that were significantly closer to those induced by plasma from hypercholesterolemic patients (yellow area in Fig. 4b). This result is significant, since it demonstrates that, unlike conventional hypercholesterolemia tests which generally measure lipid concentrations, the colorimetric lipid/PDA diagnostic assay exhibits sensitivity to lipid quality (oxidative status) as well.

To gain insight into the molecular events underlying the colorimetric transformations induced in the TM/DMPC/PDA systems by blood plasma we applied electron spin resonance (ESR, Fig. 5) for assessing the contributions of membrane interactions to the lipid/PDA diagnostics. The focus here on membrane processes is two-fold. First, lipid/PDA vesicles are generally considered an effective biomimetic membrane platform, employed for studying membrane interactions of diverse membrane-active biomolecules [11, 12]. Second, the realization that lipoproteins are the plasma components likely responsible for the colorimetric transformations (e.g. Fig. 3) points to the prominence of hydrophobic and/or amphiphilic interactions as the determinants which underlie the differences among the patient groups studied.

Figure 5 displays the rotation correlation times (τ_c) recorded in ESR experiments utilizing TM/DMPC vesicles (1:1 mole ratio) which further contained 4-(decyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT-10), an ESR probe located at the bilayer/water interface [22]. CAT-10 has been previously employed as an ESR probe for analysis of bilayer surface adsorption and dynamic effects induced by membrane-active species [23]. The ESR data in Fig. 5 indeed reveal distinct signals induced by the plasma groups examined. In particular, significantly higher average rotation

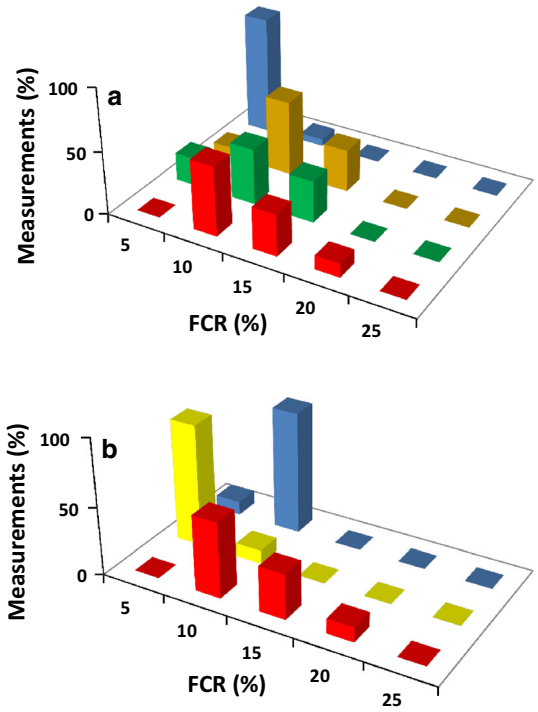

Fig. 4 Chromatic data using trimyristin/DMPC/PDA vesicle assay. Three-dimensional *bar diagrams* depicting the fluorescence chromatic response (%FCR) recorded upon incubating the high-MW fractions of plasma samples with the vesicles. **a** All samples: healthy persons (*red*), hypercholesterolemic patients (*blue*), hypercholesterolemic patients treated with simvastatin (*green*), hypercholesterolemic patients treated with simvastatin + pomegranate extract (*orange*); **b** lipid oxidation effects. Healthy persons (*red*), hypercholesterolemic patients (*blue*), the plasma from healthy persons oxidized using $CuSO_4$ (*yellow*). Oxidation shifts the colorimetric transitions towards the hypercholesterolemic profile

correlation time (τ_c) was recorded upon incubation of the CAT-10/TM/DMPC vesicles with high-MW fractions from plasma samples obtained from hypercholesterolemic patients as compared to the plasma from healthy persons. It should be emphasized that the ESR (Fig. 5) and PDA experiments (Fig. 2) probe different physical phenomena and their signals emanate from different domains within the bilayer membranes. Specifically, the PDA assay responds to bilayer surface interactions particularly closer to the polymeric PDA domains, while the ESR experiment reveals dynamic changes of the spin probe located in the vicinity of the lipid headgroups. Accordingly, it is conceivable (and the likely scenario in the experiments reported here) that the membrane-active species in healthy plasma exhibit lesser binding closer to the lipid headgroups (and thus lesser effect upon the dynamic features of the spin-probe), while the species produced in plasma of hypercholesterolemic patients tend to accumulate at the lipid headgroup environs, consequently increasing the correlation time of the ESR probe as shown in Fig. 5.

Figure 5 also indicates that high-MW plasma fractions from patients undergoing hypocholesterolemic therapy induced somewhat lower τ_c than the hypercholesterolemic patients, albeit still significantly higher than the plasmas from healthy persons. These results are consistent with the colorimetric data in Fig. 2 (sol–gel) and Fig. 4 (vesicle solutions), which demonstrated both differences in colorimetric profiles between blood plasmas from healthy versus hypercholesterolemic patients, as well as the fact that therapeutic treatment did not revert back plasma properties to the "healthy" conditions. Overall, the ESR analysis in Fig. 5 provides evidence that molecular interactions at the bilayer/water interface are a key factor contributing to the distinct colorimetric responses from the different plasma samples.

4 Conclusions

We present a new approach for screening blood plasma of hypercholesterolemic patients using a colorimetric

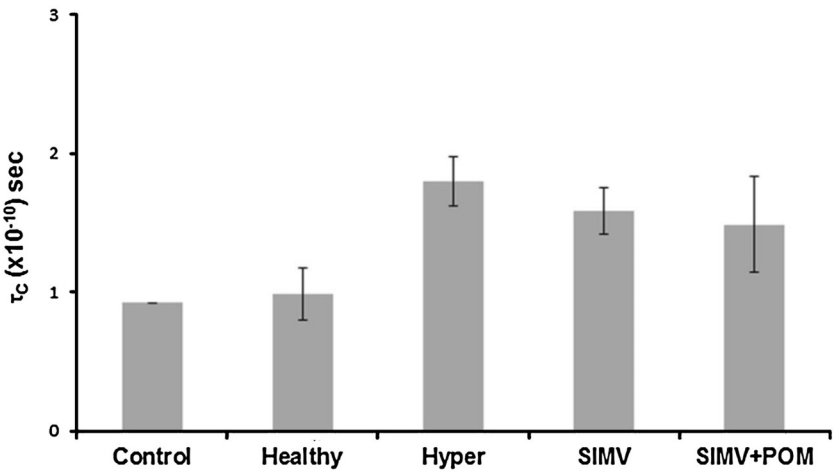

Fig. 5 Electron spin resonance (ESR) analysis. The rotation correlation time (τ_C) recorded following incubation of high-MW plasma fractions from the different patient groups with CAT-10/TM/DMPC vesicles. Significant differences are apparent between membrane interactions of plasma samples obtained from healthy individuals and hypercholesterolemic patients

diagnostics assay. The sensing system is based upon color transformations of lipid/PDA vesicles embedded within a porous sol–gel matrix, induced upon a short incubation with the plasma samples. We show that the colorimetric transitions in the gel-embedded PDA vesicle system are induced by large amphiphilic plasma particles. Importantly, the colorimetric assay could clearly distinguish between plasma samples extracted from healthy persons versus hypercholesterolemic patients. Furthermore, plasmas from hypercholesterolemic patients undergoing therapeutic treatments also gave rise to distinctive colorimetric profiles. Notably, the diagnostic assay indicates that therapeutic treatments (within the 6-week treatment period)—statins and statin + pomegranate extract, respectively—did not "revert back" the physiological condition of the patients' plasma to the "healthy" state.

Experimental analysis utilizing size-separation of plasma components and application of ESR reveals that lipid particles are likely the primary plasma constituents responsible for the colorimetric responses recorded in the lipid/PDA assays. Notably, we find that the color responses are not simply correlated to the relative abundance of cholesterol (or other lipid species) which are currently the standard diagnosis tools, but rather also reflect lipid quality (oxidation and inflammation), opening the way for using the colorimetric assay for predicting cardiovascular risk.

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Author contributions EM, SK, and RY carried out the experiments. EM and SK performed data analysis. MA obtained the plasma samples. AP, MA, and RJ directed and supervised the research and data analysis, and wrote the paper. RJ is guarantor for the overall content.

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