Dyes and Pigments 94 (2012) 156–162

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Spectroscopic investigations, characterization and chemical sensor application of composite Langmuir–Schäfer films of anthocyanins and oligophenylenevinylene derivatives

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ARTICLE INFO

Article history:
Received 13 October 2011
Received in revised form
19 December 2011
Accepted 22 December 2011
Available online 2 January 2012

Keywords:
Langmuir–Schäfer films
Anthocyanin stability
Oligophenylenevinylenes
Air/water interface characterization
UV–vis spectroscopy
Herbicide sensor

ABSTRACT

Anthocyanins, extracted from grape skin (*Vitis vinifera*), were dissolved in ultrapure aqueous subphase and an oligophenylenevinylene derivative was spread on the subphase surface. Such oligomer was chosen as anionic counterpart of hydrosoluble anthocyanins in order to perform a Langmuir–Schäfer film of the dyad. Interface interactions between oligophenylenevinylene derivative and anthocyanins were studied by Brewster angle microscopy and reflection spectroscopy. Additionally, the oligomer exhibits a chemical structure able to ensure an enhancement of the stability under UV–visible irradiations of the film of the dyad without any variation of the natural pigment absorption in the visible range. The visible spectra of cast anthocyanins film and Langmuir–Schäfer oligomer/anthocyanins film after exposure to 254 nm irradiation showed a remarkable increase of the film stability, probably due to the screening effect of the oligomer. Preliminary test of a Langmuir–Schäfer film of oligophenylenevinylene derivative/anthocyanins as a herbicide sensor showed that sensing is completely reversible, stable and repeatable.

1. Introduction

Biomaterials have the distinctiveness to work in highly organized assemblies from macro- to nano-scale. Such a peculiarity has prompted the development of multidisciplinary biomimetic approaches in modern (bio)nanotechnologies. Great effort has been dedicated to the integration of biological materials with synthetic substances to design and realize architectures constituting a large set of different functional elements [1]. The large availability of coloured bacteria, flowers, fruits and leaves permits the extraction of different anthocyanins suitable for utilisation in functioning devices. Therefore they represent a commonly encountered natural class of compounds and additionally their characteristic interaction with radiation renders them very appealing biomaterials. In fact these biomaterials are non-polluting, fully-biodegradable, low-cost, natural, and non-toxic and consequently can successfully replace other artificial pigments, usually expensive, difficult to synthesise and not with ceaseless and permanent availability. Moreover anthocyanins exhibit a broad absorption band in the visible region due to HOMO-LUMO charge-transfer transition; the corresponding energy separation between 2.2 and 2.4 eV, equivalent to peaks in the region 560-520 nm; this visible absorption has enabled anthocyanins to be investigated as reagents/analytes by absorption spectroscopy.

Anthocyanins are polyphenols that occur widely in fruits, flowers and other plant tissues and are invariably found as glycosides of the anthocyanidin aglycone form, which comprises cyanidin, delphidin and pelargononidin sharing a common chemical structure and differing only in the number and position of hydroxyl groups on the B-ring (Fig. 1).

More than 600 different anthocyanins, with a variety of substituents, have been reported [2]. Based on studies on relatively simple anthocyanins, a scheme of their structural changes related to pH values has been proposed. Accordingly, at pH 3 or lower, the orange, red or purple flavylium cation is predominant and is responsible for the characteristic colour of many fruits. At higher pH values, kinetic and thermodynamic competition occurs between the hydration reaction on position 2 of the flavylium

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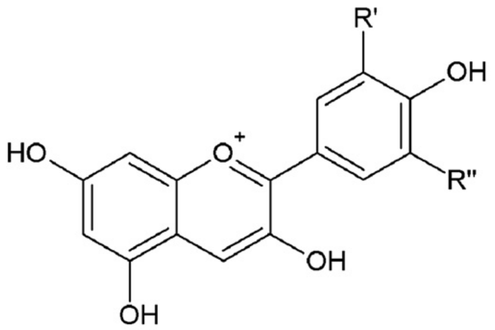
doi:10.1016/j.dyepig.2011.12.010



Grape's anthocyanins	R'	R''
malvidin	$-OCH_3$	$-OCH_3$
delphinidin	-OH	-OH
petunidin	-OH	$-OCH_3$
cyanidin	-OH	-H
peonidin	$-OCH_3$	-H

Fig. 1. General anthocyanin structure.

cation and the proton transfer reactions involving its acidic hydroxyl groups. While the first reaction at the flavylium cation [3] gives a colourless carbinol pseudobase, which can undergo ring-opening to yellow chalcones, the proton transfer gives rise to the purple quinonoidal base. Further deprotonation at pH 6–7 results in the formation of more bluish resonance-stabilised quinonoid anions. At acidic pH values, typical for fresh and processed fruits and vegetables, each anthocyanin will be as a mixture of equilibrium forms.

One of the problems connected with the employment of anthocyanin pigments in the use of these materials in electronic devices such as biosensors or dye sensitized solar cells is their stability, since they are often degraded during processing or storage. Their degradation can proceed by either of two pathways to form chalcone and coumarin glycosides or aldehydes and benzoic acid derivatives [4]. A number of physical and environmental factors affect anthocyanin stability. Among these, temperature (degradation rate increases with rising temperatures), UV-light and pH. Low pH values stabilise anthocyanins, since the equilibrium between the coloured flavylium cation and the colourless pseudobase is shifted towards the former more stable compound. It was shown that tart cherry anthocyanins were rapidly degraded to benzoic acid, when the pH increased from 7 to 10 by addition of 1 N KOH [4]. Singlet oxygen is able to degrade meoru (*Vitis coignetiae*) anthocyanins in aqueous solution, suggesting a quenching process of singlet oxygen by this class of compounds. The same authors showed that diglucoside anthocyanins are more stable than the monoglucoside and that the number of hydroxyl groups in the structure is important in the compound stability under singlet oxygen, with malvidin glucoside being more stable than delphidin or cyanidin glucoside [5].

2. Experimental

2.1. Deposition method and analyte's choice

Differently, conjugated polymers and oligomers have attracted great interest in recent literature from both a fundamental point of view and an applied science perspective: their electrical, luminescent, and photoconducting properties make them suitable for applications in optoelectronic devices [6,7]. Among conjugated polymers, poly(*p*-phenylenevinylene) (PPV) derivatives combine a reasonably good environmental stability to high optoelectronic properties [8], and are viewed as active components for several organic devices.

Anthocyanins are utilized in solution [9], or on solid supports [10] but the low stability and short lifetime of devices are limiting aspects for the development of anthocyanin-based technology. Polymer films may be fabricated using several techniques, including the Langmuir–Blodgett (LB) method or the horizontal lifting Langmuir–Schäfer (LS) method whereby nanostructured films may be achieved with controllable thickness, low number of defects, and some degree of organization at the molecular scale [11]. Using such films is therefore attractive, since their homogeneity and organization may affect positively the optical and electrical properties of devices. The LB technique also has the advantage of requiring a very small amount of precious material, in contrast to the other techniques such as spin coating or casting. The latter methods, even though quick and cheap, do not ensure any thickness control of transferred layers [12]. The LB method is a suitable procedure for transferring thin films of amphiphilic substances from the air–water interface onto different substrates such as glass, ITO, quartz, gold, Mylar and SiO_2 [13,14]. Monitoring the surface pressure of the floating layer vs. the average area per molecule permits the identification of maximum packing conditions and promotes the formation of thin films with highly reproducible characteristics. Unfortunately, not all molecules can be processed into single-component multilayers by this technique. The appropriate materials have to preferentially possess the proper balance between hydrophilic and hydrophobic properties and between rigid and flexible moieties to facilitate the formation of stable floating monolayer phases [11]. In earlier reports, most LB films of PPVs were prepared using heat treatment of LB films of precursor polymers [15]. No investigations have been reported about the deposition of LB films of anthocyanins. The main drawback of this method is the typically long time needed for the film fabrication even though the LS method allows more rapid transfers.

It is well-known that anthocyanins have hydrophilic nature and this prevents their transfer by the LB method, even though the positive charge on the anthocyanins allows the assembly of supramolecular systems coupling the flavonoids with LB transferable negatively charged species. So in our investigations the driving force promoting film formation arises mainly from electrostatic interactions between positively charged water soluble anthocyanins and negatively charged amphiphilic oligophenylenevinylene. The final organization in LS films depends on this electrostatic approach, already used for the fabrication of different multilayers [16]. In order to evaluate the effect of the oligophenylenevinylene (FPPV), namely the sodium 3-{2,5-bis[*E*]-1,2-difluoro-2(-4-octyloxyphenyl)vinyl]-4-methoxyphenoxy}propane-1-sulfonate, as a screen for anthocyanine molecules a LS film of the dyad anthocyanine/FPPV has been exposed to 250 nm radiation. The pH effect on the fabricated films has been checked in preliminary investigations as active layers for the detection of a common plant growth hormonal active herbicide, Erbitox E30, a commercial form of MCPA (2-methyl-4-chlorophenoxyacetic acid, Fig. 2) a systemic phenoxy alkaline herbicide used to control annual and perennial weeds in cereals, grasslands, trees and turf.

The use of the FPPV allowed employment of the solid thin film of anthocyanins as active layer for hydrosoluble analytes (as MCPA) with a simultaneous increase of the device stability. Phenoxy herbicides are absorbed through leaves and then concentrated in the meristematic tissues, where their toxic activity is carried on interfering with protein synthesis, cell division and ultimately with the plant's growth [17]. The mechanism of MCPA toxic action is not yet well-understood. The compound causes skin and mucosa irritation. Furthermore, MCPA absorption could give renal, hepatic, myocardial and nervous lesions; these herbicides have also teratogenic effects [18], and the presence of impurities, such as dioxins and dibenzofurans, can bring to immunosuppressive and enzymatic induction activity. There is also the likelihood of a carcinogenic effect, even if such opinion is not completely shared among researchers [18].

2.2. Materials and procedures

Anthocyanins were extracted from grape skin (*Vitis vinifera*, cv. Primitivo), previously lyophilized and frozen. The grape skin was in the presence of liquid N_2, thus providing a uniform powdered sample. A powder sample (5 g) was used for the extraction with methanol (50%) - water (48.5%) - formic acid (1.5%), and incubated and stirred for 30 min at 25 °C. The extract was centrifuged at 5000 rpm for 10 min and the supernatant was dried by a rotary evaporator under vacuum to permit solvent evaporation [19].

The extracted anthocyanins were characterized by a chemical-physical point of view, another part was dissolved in the mobile phase for HPLC analysis and fractionated on a reverse phase analytical column (C18 type Ultrasphere 5 μm Spherical 80 Å pore, 25 mm) for HPLC analysis on by a chromatography workstation 1100 Agilent equipped with a UV–Vis photodiode array detector. Chromatography was developed by a linear gradient of increasing acetonitrile concentration in acidified water (from 0 to 60 % in 35 min), and the elution of individual compounds was monitored at 520 nm. Anthocyanins were identified by comparison of the retention times and spectra of authentic chemically synthesized standards (Extrasynthèse®) and a chromatogram is reported in Fig. 3.

Synthesis and purification of the oligophenylenevinylene FPPV were performed according to the procedure reported in the literature [20].

Anthocyanins/FPPV dyad films were deposited onto solid supports by a KSV 5000 LB apparatus (850 cm^2). Ultrapure water (Millipore Milli-Q, 18.2 MΩ cm) was used as the subphase and for dissolving anthocyanins. Subphase temperature was kept at 20 °C during the experiment. FPPV was dissolved in $CHCl_3$ and the solution was spread on the subphase. After solvent evaporation, the barriers of the trough were moved at 10 mm min^{-1}; the surface pressure was simultaneously monitored by a Wilhelmy balance and the isotherm of the spread film was recorded. Floating films were transferred onto a quartz substrate, hydrophobized by exposure for 24 h to saturated atmosphere of hexamethyldisilazane, by the Langmuir-Schaefer (LS) procedure [21].

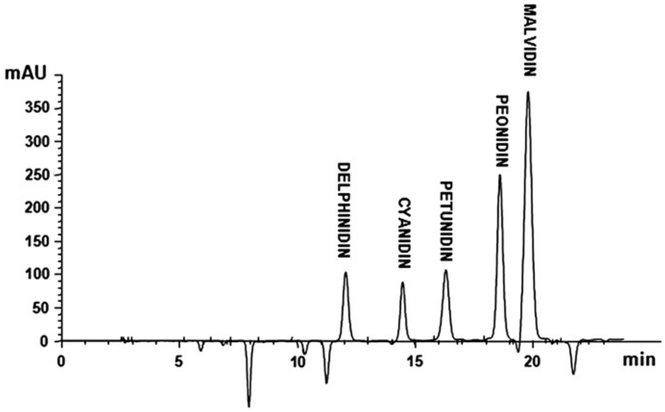


Fig. 3. HPLC profile of extracted anthocyanins from grape skin (*Vitis vinifera*).

Brewster Angle Microscopy (BAM) experiments during floating film compression were performed by a NIMA 601/NFT BAM2plus apparatus with a lateral resolution of 2 μm. Reflection spectra were recorded by means a NFT RefSpec Instrument. A compression speed of 7 $Å^2$ $molecule^{-1}$ min^{-1} was utilized, after evaporation of chloroform. They were acquired at 293 K under normal incidence of radiation. Visible measurements on solid supports were carried out by means a Agilent Cary 5000 spectrophotometer.

3. Results and discussion

3.1. Floating film characterization

FPPV (Fig. 4) was chosen as the anionic counterpart to the hydrophilic anthocyanines in the deposition of a thin film using the LS approach. The oligophenylenevinylene structure functionalized with fluorinated double bonds, alkoxy end chains and a propyloxy sulfonate group, makes FPPV an amphiphilic compound suitable for LS transfer. Fluorination of vinylene units was accomplished to improve the chemical stability of the material. Substitution of carbon-hydrogen bonds with stronger and less reactive carbon-fluorine bonds increases the resistance to the photooxidation processes.

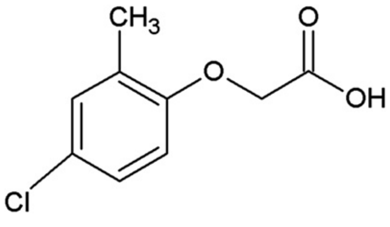


Fig. 2. 2-methyl-4-chlorophenoxyacetic acid (MCPA) structure.

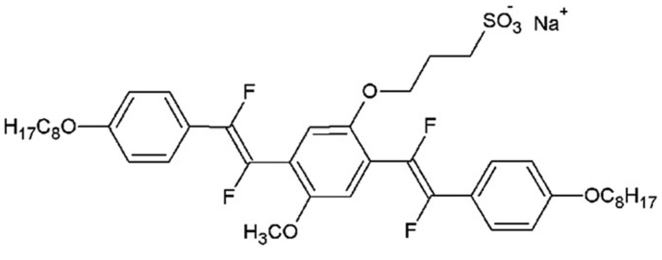


Fig. 4. Chemical structure of the oligomer FPPV.

The oligomer FPPV was dissolved in chloroform (0.25 mg/ml): 30 μl of the solution were spread on the subphase at pH 3.5 (value set adding HCl to the subphase).

After the solvent evaporation, the isotherm curve Π *vs* area per molecule of FPPV (dotted line in Fig. 5A) was recorded. A wide gaseous-like phase can be observed for values of area per molecule down to 150 Å^2; then a quick variation of the curve slope is registered and the oligomer moieties are organized in the liquid like phase. A further compression induces another slope variation at about 15 mN/m. The increase of Π is usually associated with a closer packing of molecules. The limiting area per molecule of FPPV, obtained by the extrapolation of the steepest portion of the Langmuir isotherm at $\Pi = 0$, is about 90 Å^2.

When anthocyanins were dissolved in the water subphase with a concentration of 0.22 g/l, the pH value resulted was 3.5; compression of the barriers on the bare subphase did not induce any pressure variation. At high values of area per molecule the isotherm registered spreading 30 μl of FPPV (0.25 mg ml^{-1}) appears very similar to the isotherm recorded when the oligomer is spread on pure water subphase. Upon compression the curve Π vs area per molecule of FPPV evidences an onset for surface pressures at the same value recorded for FPPV on the water subphase at pH = 3.5 without any dissolved anthocyanin (continuous line in Fig. 5A). The Langmuir curve pattern varies in comparison with the previously obtained curve and the limiting area increases to 180 Å^2. These observations suggest that the presence of the anthocyanins in the subphase, at high Π values, prevents the oligomer aggregation and such behaviour can be explained supposing that anthocyanins in the subphase electrostatically interact with the negatively charged oligomer FPPV spread on the subphase, forming a supramolecular dyad.

Images of the floating film acquired at the Brewster angle during compression confirm the previous rationale derived from the comparison of isotherm curves. Fig. 5B and C evidence that oligomer FPPV, spread on water at pH 3.5, forms small shining tridimensional aggregates even at low surface pressures (9 mN/m); their density and dimension increase when the area between the two barriers decreases (Fig. 5C, registered at 23.5 mN/m). The π-π stacking interaction is probably more energetic than the interaction of the anionic sulfonate group with the polar water molecules; moreover, according to the comments reported on different PPV derivatives [22], the absence of a uniform floating layer can be ascribed to the lack in its structure of a suitably long alkoxy functionalisation.

On the contrary, the floating film of FPPV on the anthocyanin-containing subphase seems to be uniform at very low pressure values and for Π larger than 5 mN/m the morphology does not show significant variations (Fig. 5D and E). Also this behaviour is consistent with an effective electrostatic interaction among the oppositely charged constituents of the composite layer. A plausible rationale is that the coulombic interaction between the anionic sulfonate group of FPPV and the cationic termination of anthocyanins is capable to prevent the formation of the π-π stacks of FPPV observed when the dye moieties were not dissolved in the subphase; this also implies an increase of the area per molecule in the surface pressure vs. area Langmuir curve as actually observed in Fig. 5A.

The stability of the two different floating layers (with and without anthocyanins dissolved in the subphase) has been investigated by compressing the floating films at a surface pressure of 25 mN/m and contemporaneously permitting the barrier movement in order to keep surface pressure constant. The observations confirm the improved stability induced by anthocyanins in the subphase: in fact the decrease in the area per molecules over 3 h was 18% without anthocyanin dyes and only 5% with anthocyanin dyes in the subphase.

Reflection spectroscopy (RefSpec) carried out at the air–subphase interface contributes further evidence of the interaction between FPPV and hydrosoluble anthocyanins. Such a technique allows the evaluation of the difference between the reflectivity of the bare subphase and the subphase covered by the floating film (ΔR). It has been demonstrated that ΔR is directly proportional to the absorbance of the cromophores placed exclusively at the interface [23]. Fig. 6 shows a band at 547 nm (continuous spectra) even for low values of surface pressure. This band can be ascribed to the anthocyanin absorption comparing the reflection spectra with oligomer (Fig. 6, dotted line) and anthocyanins solutions (dashed line in Fig. 6A).

It is noteworthy that the oligomer absorption maxima at 304 and 342 nm do not shift, but the anthocyanin peak is significantly red shifted by 30 nm indicating strong electronic interactions between the oligomer and the anthocyanins.

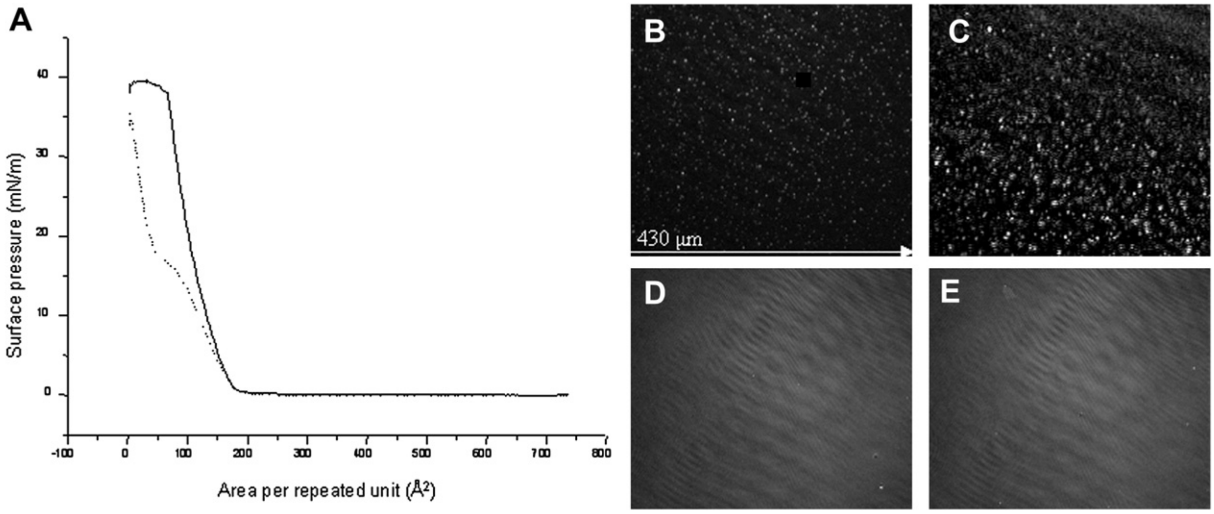


Fig. 5. (A) Isotherm curves for FPPV on water subphase at pH 3.5 (dotted line) and for FPPV/anthocyanins system (continuous line). In both cases an aliquot of 30 μl of FPPV was spread and the subphase pH was kept at 3.5; BAM images of FPPV on water subphase at pH = 3.5 and acquired at surface pressure values of (B) 9 and (C) 23.5 mN/m; BAM images of FPPV spread on subphase of anthocyanins dissolved in water in concentration 0.22 gr/L (pH = 3.5) acquired for increasing values of surface pressure: (D) at 6 mN/m and (E) 10 mN/m.

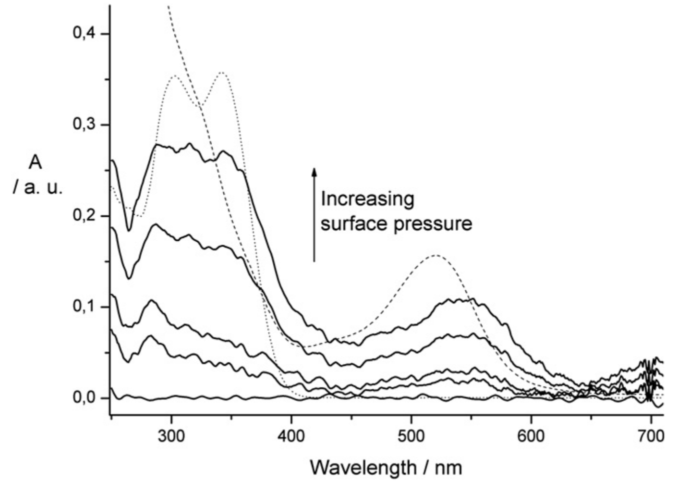


Fig. 6. Floating film spectra of FPPV on anthocyanins solution subphase (continuous lines), dashed and dotted line represents, respectively, visible spectrum of anthocyanins dissolved in water and FPPV dissolved in $CHCl_3$.

3.2. Stability tests on LS film of dyad oligomer/anthocyanins

The stability of anthocyanins in aqueous solution has been studied as a function of pH [24], temperature [25], and other parameters such as the presence of endogen enzymes and other substituents [26]. Another cause of anthocyanins instability derives from to the effect of UV radiation, and it represents a limiting aspect for using such substances as photoactive material for solar cells and dye synthesized solar cells (DSSC) construction. Examples of DSSCs where anthocyanins, extracted from blackberries, are employed as dye are reported with incident photon-to-current efficiency of 19% [27]. Moreover solar devices made using red Sicilian orange juice with a solar energy conversion efficiency up to 0.66% were constructed and characterized. Here the cells degrade in a week if stored in the dark and at a temperature of 5 °C and within few hours at room temperature under UV–vis irradiation [28a,b]. To improve the stability of extracted anthocyanins we transferred the floating film by LS technique, at a surface pressure of 22 mN/m. The visible spectrum of FPPV/anthocyanine layers (20 LS runs) shows that the anthocyanins absorption maximum when transferred with FPPV on the solid support is localized at the same wavelength observed at the air–subphase interface and, as a consequence, it is

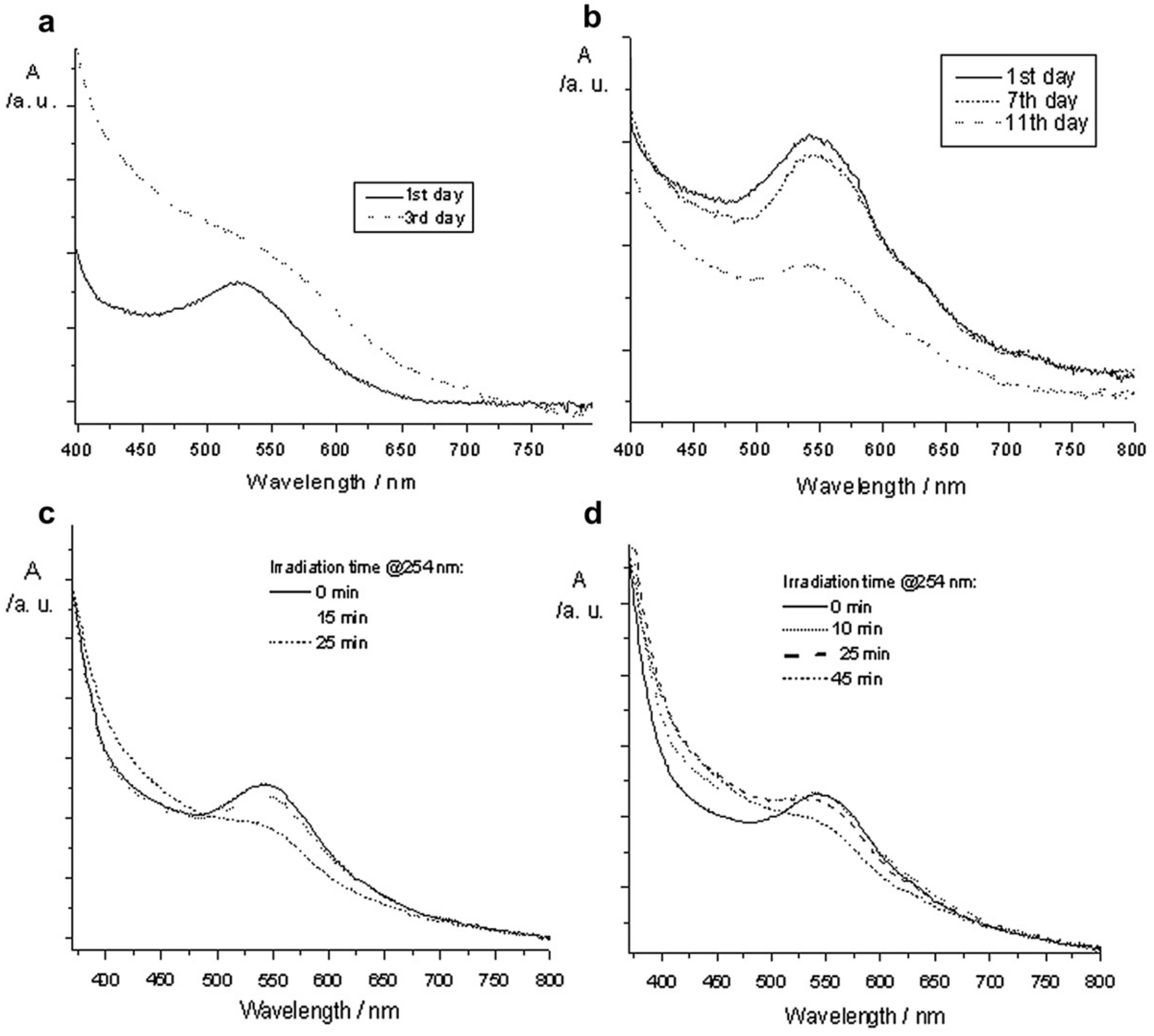


Fig. 7. (a) Monitoring of the visible spectrum of the aqueous solution of anthocyanins during the days; (b) temporal evolution of the visible spectrum of the film (20 LS runs) of the dyad FPPV/anthocyanin; (c) effect induced from the UV radiations on a FPPV/anthocyanins//10 FPPV LS film and (d) on a FPPV/anthocyanins//20 FPPV LS film.

red shifted in comparison with the solution spectrum (Fig. 1S, supporting contents).

The stability of the extracted anthocyanins in aqueous solution at pH = 3.5, as a cast film on a quartz sample and as FPPV/anthocyanins LS film, was compared recording the temporal evolution of their visible spectra and storing them at 25 °C under solar radiation. The aqueous solution showed some colour variation in just one day, and three days were enough to observe strong variations in the spectral profile (Fig. 7a). The cast film, obtained by the evaporation of a drop of aqueous solution of anthocyanins on a quartz substrate, appeared stable up to three days; after a week the absorption peak was red shifted by 10 nm and a strong decrease of absorbance was recorded; moreover a shoulder at 577 nm rised up (data showed in Fig. 2S, supporting contents).

LS films of FPPV/anthocyanins show a sensible improvement of the dye stability: visible spectra did not exhibit any variation up to six days, then the absorption intensity decreased (Fig. 7b).

To evaluate if the oligomer merely works as UV photons screen, a cast film of anthocyanins, two FPPV/anthocyanins LS films covered with 10 and 20 LS runs of FPPV (from here on, respectively, FPPV/anthocyanins//10 FPPV and FPPV/anthocyanins//20 FPPV) and a LS film of FPPV (20 runs, $\Pi_{deposition} = 16$ mN/m) were exposed to UV irradiation (254 nm, lamp power 30 W) for different intervals of time. The cast films of anthocyanins showed a strong decrease of maximum value of absorbance in 10 min, and a complete bleaching in 15 min as showed in Fig. 3S (supporting contents).

The same exposure time was not long enough to induce a remarkable variation in the spectral profile of FPPV/anthocyanins//10 FPPV LS sample, but further exposure (up to 25 min) provoked a change in the absorption bands of the sample (Fig. 7c).

The screening effect of FPPV should be enhanced increasing the number of layers deposited on the dyad's film. Visible spectra recorded on the sample FPPV/anthocyanins//20 FPPV appeared unchanged if illuminated for more than 25 min even if strong variations for lower wavelengths can be observed (Fig. 7d). Such experimental evidences suggest that FPPV absorbs UV photons changing its spectra and protecting the anthocyanins. The degradation of FPPV under 254 nm light is reported in Fig. 4S (supporting contents), with the variation of signals of the oligomer LS film after 30 min exposition to UV radiation.

All these investigations evidence the function of the oligomer layer as a protecting screen towards UV radiation.

3.3. *pH variations induced in the LS film of FPPV/anthocyanins dyad and potential applications as herbicide sensor*

Anthocyanins are well-known pH indicators, a difference in chemical structure occurs which is dependent upon pH variations and the absorption maximum shifts from red region in acid environment towards blue at a basic pH [24]. We also tested such behaviour on our LS films. We observed that the deposited LS layers FPPV/anthocyanins are not water soluble (no bleaching of maximum absorption is recorded when the sample is dipped in water). The stability of anthocyanins in the composite film has prompted us to carry out initial measurements using the LS films as the active layers in chemical sensors for the detection of hormone type herbicides, usually operating at basic pH values. Here, just as an example of potential applications, a FPPV/anthocyanins//20 FPPV LS film was deposited on a quartz substrate and it was incorporated in a home-made flow cell [29]. An aqueous solution (0.3 g/L) of Erbitox E30 was fluxed on the sample and, simultaneously, the absorption spectra were acquired. After the sample exposition to analyte, HCl aqueous solution at pH = 4 was fluxed on the solid sample. Spectra before, during and after analyte – LS film interaction are reported in Fig. 8. The herbicide induces both a quick red shift and an almost complete bleaching of the absorption peak (bold continuous line in Fig. 8), and the sensing process is completely reversible (dotted line) when water at pH 4 is fluxed in the cell for few minutes. Cyclic injections of herbicide aqueous solution were repeated and the reversibility of chromatic change was observed. A bleaching of the absorption spectra were recorded overcoming 15 cycles.

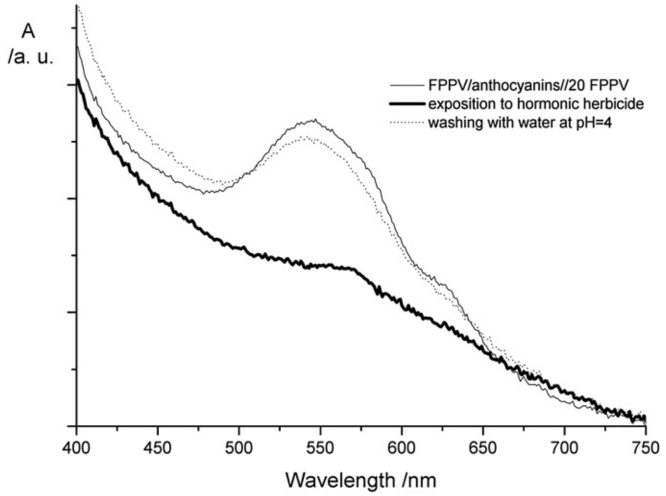


Fig. 8. Variation of the visible spectrum of the LS sample FPPV/anthocyanins//20 FPPV during exposure to a solution of a commercial hormone type pesticide (Erbitox E30).

4. Conclusions

Composite films of positively charged water soluble anthocyanins and a negatively charged fluorinated oligophenylenevinylene derivative were transferred onto solid substrates. The electrostatic interaction between the two components is energetic enough to promote the transfer of both moieties by the LS method. Evidences of such effective interaction at the air–water interface have been obtained by Π vs. area per molecule curves, Brewster Angle Microscopy and UV–Vis reflection spectroscopy. The presence of the oligomer in the film induces improved stability of the anthocyanin moieties when exposed to UV–Vis radiations. Preliminary investigations on the use of these multilayers in colourimetric chemical sensors for the detection of a commercial herbicide have been successfully performed.

Acknowledgement

The authors gratefully acknowledge Mr. Luigi Dimo for his technical assistance and Mr. Leone D'Amico for anthocyanins HPLC analysis.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dyepig.2011.12.010.

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