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# Phospholipase activity is modulated by c-Fos through substrate expansion and hyperpolarization

Graciela A. Borioli*, Beatriz L. Caputto, Bruno Maggio

*CIQUIBIC, Departmento Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba Pabellón Argentina, Ciudad Universitaria, 5000 Córdoba, Argentina*

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**Abstract c-Fos, a component of AP-1 transcription factors, has been shown to have marked amphitropic properties and to regulate phospholipase activity against lipid monolayers. In agreement with its high surface activity, it has also been found to associate to membranes of the endoplasmic reticulum and to activate phospholipid metabolism in vivo. All these findings point to an involvement of this oncoprotein within a membrane environment. We have previously shown that c-Fos modulates in different manners the activity of phospholipase $A_2$ and phospholipase C against monolayers of dilauroylphosphatidylcholine (PC). In this work, we have studied the possible molecular mechanism underlying the phosphohydrolytic modulation. Our results show that c-Fos expands and hyperpolarizes PC, indicating that its effects on these enzymatic activities are due to the changes it induces on the interfacial organization of the substrate.**



*Keywords:* c-Fos; Phospholipase activity; Dielectric polarization vector; PC; Surface molecular packing

## 1. Introduction

The amphitropic properties of c-Fos, which was formerly considered a soluble protein, have been well-established [1]. c-Fos is linked to phospholipid metabolism through its ability to activate synthesis [2] in a variety of systems, and to modulate phospholipases $A_2$, C and sphingomyelinase activities against lipid monolayers [3]. In these studies, we found that the effect of c-Fos was different on each enzymatic activity in a manner that depends markedly on the lateral surface pressure [3]. This capacity of c-Fos, together with its ability to interact differentially with various phospholipids [1], called for studies directed to elucidate a possible molecular mechanism for its effects on the enzyme activities at the membrane level. Phospholipases are a rather heterogeneous group of enzymes but have remarkable common properties regarding their extreme sensitivity to a few interfacial parameters that control the intermolecular organization of the substrate [4]. Among the many factors shown to regulate the activity of all phospholipases, the packing state, phase state and composition of the membrane are crucial [5–14]. An obvious phenomenon to explore when attempting to elucidate activity regulation is the dynamics of protein–lipid interactions that underlie many effects of amphitropic proteins on membrane events [7,13,15–17], including phospholipid degradation by phospholipases. Thus, we deemed it worth to look at the nature of the interactions of c-Fos with PC, a substrate for both $PLA_2$ and PLC, in our search for clues about phospholipase modulation by c-Fos. We have analyzed the compressional behavior of lipid-protein mixtures with different proportions of protein, spanning the relatively small amounts of c-Fos that are able to exert an effect on enzymatic activities in monolayers.

## 2. Materials and methods

Recombinant N-terminal histidine tagged c-Fos was purified by affinity chromatography [1]. Langmuir monolayers of premixed binary mixtures of c-Fos and dilauroylphosphatidylcholine (PC) (Avanti Polar-Lipids, Inc., Alabaster, AL) or of the pure components in chloroform–methanol–$H_2O$ 2:1:0.3 were formed and their surface parameters measured [1,18], by spreading less than 50 μl of lipid–protein solution on the surface of 74 ml of 145 mM NaCl in a 80 $cm^2$ circular teflon trough compartment (Monofilmmeter, Mayer Feintechnik, Germany). Surface pressure was measured by a platinized-Pt sensing plate connected to a surface pressure transducer. Surface potential was measured by a high impedance millivoltmeter connected to a surface ionizing $^{241}Am$ electrode positioned 5 mm above the monolayer surface, and to a reference calomel electrode connected to the aqueous subphase through a saline bridge. Absence of surface-active impurities in the subphase and in the spreading solvents was routinely controlled as described previously [18]. The monolayers of the pure components and of all mixed films were stable and reproducible, with hysteresis, after several compression–expansion cycles. Less than 10% of molecules were lost from the interface during those cycles as indicated by negligible variations of the limiting mean molecular area and dipole potential at the collapse pressure. Deviations of mean molecular area and surface potential per unit of molecular surface density from the additive behavior were ascertained as published elsewhere [18]. At least duplicate monolayer isotherms, which were stable and reproducible after several compression–expansion cycles, were obtained and averaged at a compression rate of 0.45–0.60 $nm^2\,molecule^{-1}\,min^{-1}$; reducing the compression speed produced no change in the isotherms. Reproducibility was within maximum S.E.M. of ±1 mN/m for surface pressure, ±30 mV for surface potential, and ±0.04 $nm^2$ for molecular areas. For the analysis of the polarization vector, some data are used from previous determinations of phospholipase activity. Both $PLA_2$ and PLC activi-

* Corresponding author. Fax: +54-351-4334074.
*E-mail address:* gborioli@dqb.fcq.unc.edu.ar (G.A. Borioli).

*Abbreviations:* PC, dilauroylphosphatidylcholine; $PLA_2$, porcine pancreatic phospholipase $A_2$; PLC, *B. cereus* phospholipase C; EFM, Epi-Fluorescence Microscopy; BAM, Brewster Angle Microscopy

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ties were determined as described elsewhere [3] under zero-order catalytic reaction, after the pre-catalytic lag-time period, in real time by using PC as substrate with continuously controlled molecular packing; in these conditions, soluble products are formed that are immediately desorbed from the interface. The dielectric polarization vectors $P$, representing the energy required in ordering the molecular dipole moment on the lipid molecular area in the direction perpendicular to the interface, were calculated as previously reported [19] from the ratio between the surface free energy per molecule and the surface potential per unit of molecular surface density ($P = \mu_{N^0}/\Delta V/n$, with $\mu_{N^0} = 2\gamma A$ and $\Delta V/n = \Delta VA$, where $\gamma$ is the surface tension, $A$ is the molecular area, $\Delta V$ is the surface (dipole) potential and n is the number of resultant molecular dipoles per unit of lipid area). The values of $\mu_{N^0}$ and $\Delta V/n$ are calculated with data taken from the isotherms of surface pressure and surface potential versus molecular area obtained in this work for mixed monolayers of c-Fos-PC, and from a variety of films of PC–ganglioside derivatives and PC–sphingosine mixtures in which the phospholipase activities were previously studied [19].

Monolayers for simultaneous observation by Brewster angle microscopy (BAM) (miniBAM, Nanofilm Technologies, Göttingen, Germany) and Epi-fluorescence microscopy (EFM) (Zeiss Axiovert, Carl Zeiss, Oberkochen, Germany) were spread from the same premixed solutions with 2% egg NBD-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl, Avanti Polar Lipids, Inc.) over the surface of a rectangular 332 $cm^2$ teflon trough (KSV minithrough, KSV Helsinki, Finland) with 150 ml of 145 mM NaCl; NBD-PE is a fluorescent probe that preferentially partitions into surface phases having a liquid-expanded character [20,21].

## 3. Results

### 3.1. Analysis of isotherms

The analysis of deviation from ideality of mixed PC-c-Fos monolayers clearly evidences mean molecular area expansions and hyperpolarizations that are more marked at low surface pressures, that is, in expanded interfaces (Fig. 1).

The variation of the partial molecular parameters with the film composition, calculated by the method of intercepts [18], helps to understand the contribution of each component to these deviations from ideality, which depends on the composition of the mixture. A small proportion of c-Fos results in a large expansion and hyperpolarization of the protein exclusively, whereas PC accounts for these effects as the amount of c-Fos in the mixture increases (Fig. 2).

When one of the components deviates from ideality, the other remains unaltered. Also, the magnitude of deviations decreases with the surface pressure and becomes almost negligible in films with the highest proportion of protein at 30 mN/m. The overall effect is that, as each component is more resilient to the influence of the other at high surface pressure, the mixed films behave closer to ideality under such conditions. The partial mean molecular area and surface potential/molecule of c-Fos, when its proportion in the mixture is above 5 mol%, remain similar to those of the pure protein. Monolayers of the pure protein undergo two surface pressure-induced changes of packing and dipolar organization, one at about 13 mN/m and another at 32 mN/m, before film collapse at 46 mN/m [1]. In mixtures with PC, the two-dimensional surface pressure–composition phase diagram (not shown) indicates that the first protein reorganization remains unaltered, independently on the lipid–protein proportions in the film, while the presence of hyperpolarized PC in the expanded packing state induced by the protein impairs the adoption by the latter of the more condensed organization that it normally shows in pure protein films at 32 mN/m.

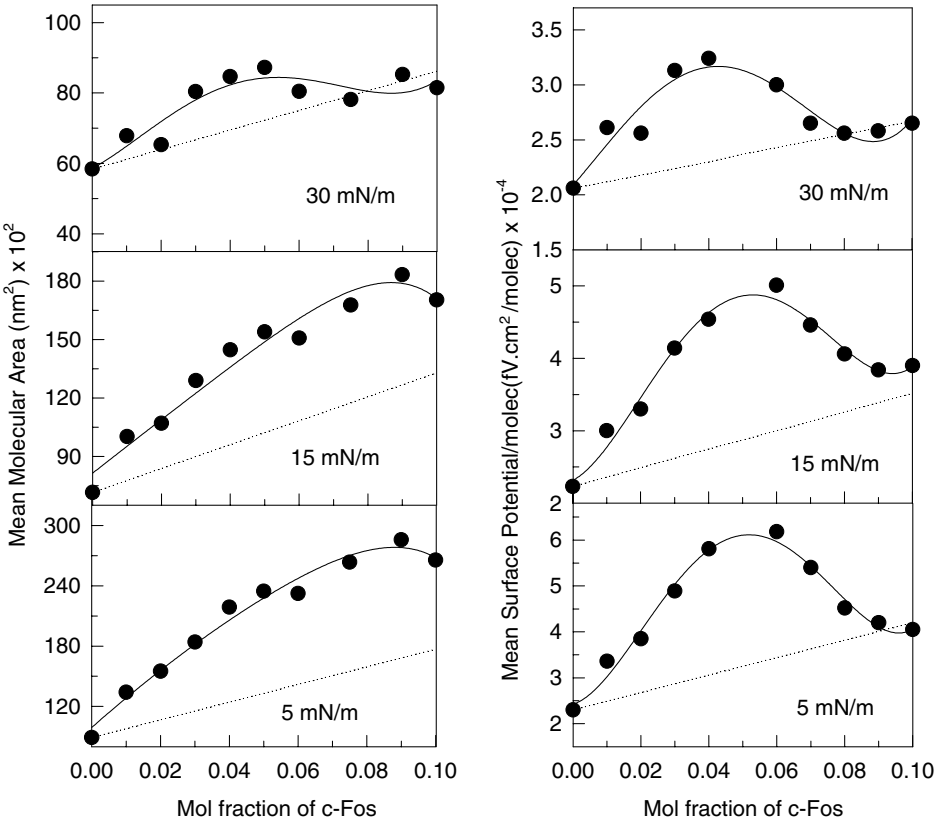


Fig. 1. Deviation from ideality of mean molecular area (left panels) and mean surface potential/molecule (right panels), of mixtures of PC and c-Fos as a function of composition at the indicated surface lateral pressures. The dotted lines represent ideal mixing behavior.

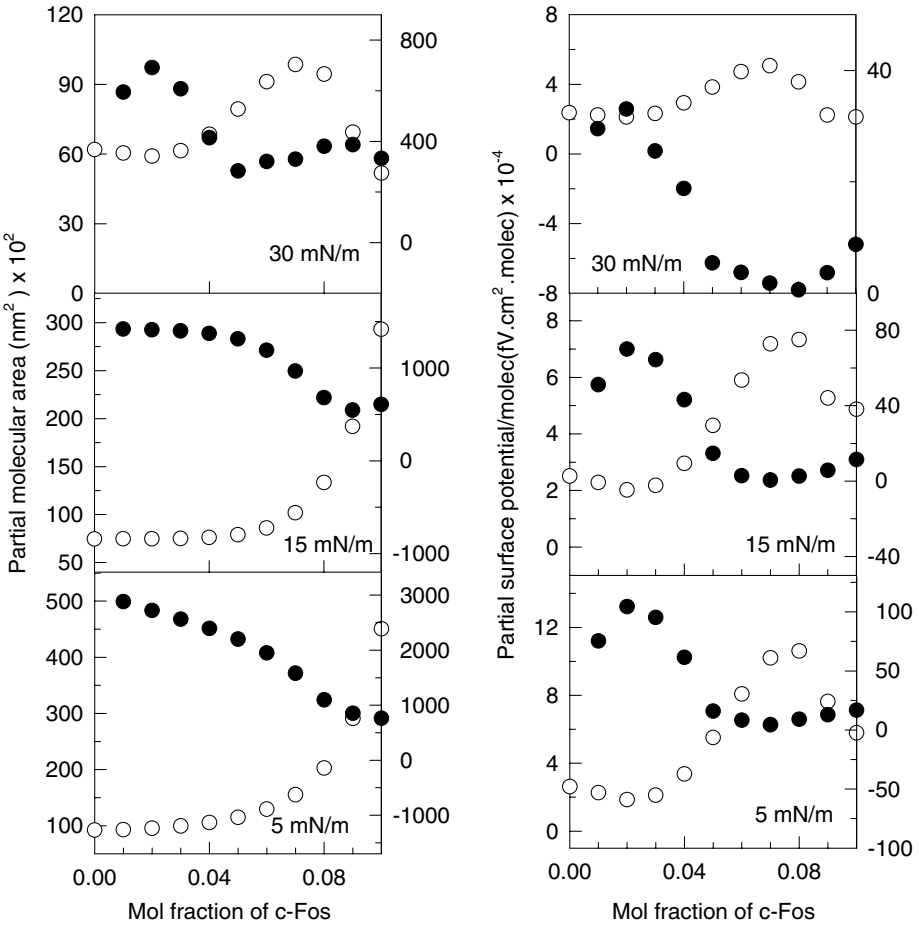


Fig. 2. Partial mean molecular area (left panels) and partial mean surface potential/molecule (right panels) as a function of the composition for mixed films of PC and c-Fos at the indicated surface pressure. The points show the variation of the partial molecular parameters of PC (empty circles) or c-Fos (black circles) in the mixed monolayers. The mean molecular area and mean surface potential/molecule of pure c-Fos are 9.68 nm$^2$ and 21.34 fV cm$^2$/molecule at 5 mN/m, 6.86 nm$^2$ and 15.09 fV cm$^2$/molecule at 15 mN/m, and 3.37 nm$^2$ and 8.16 fV cm$^2$/molecule at 30 mN/m. Left scales on each panel correspond to the lipid and right scales correspond to c-Fos.

### 3.2. Polarization of the interface and phospholipase activity

Table 1 shows data reported in our previous work [3] for the lag-time period and steady-state rate of activity of $PLA_2$ and PLC against pure PC or mixed films of PC with 8 mol% of c-Fos.

In view of the observed hyperpolarization of mixed films, we analyzed the correlation of substrate polarization with the catalytic steady-state phase and the lag time period of the phosphohydrolytic reactions. For this analysis, we have also included data re-elaborated from previous work [19]. Fig. 3 shows the variation of those reaction steps affected by a variety of compounds in mixed films with PC [19] with changes of the substrate's dielectric polarization vector, which is inversely related to the polarization of the interface. The reaction rate of $PLA_2$ (Fig. 3A) increases by a fourth as the dielectric polarization vector of PC in mixed films decreases from 100 to 20 μC/cm$^2$. In addition, the lag-time decreases four times (Fig. 3B) for the same decrease of dielectric polarization vector, so that the effect on the reaction as a whole includes both a similar (for values of dielectric polarization vector below 40 μC/cm$^2$) or a faster precatalytic activation and a higher steady-state activity of $PLA_2$ as the substrate's dielectric polarization vector de-

Table 1
Phospholipase activity of monolayers of PC with c-Fos

| Enzyme | Surface pressure (mN/m) | Lag time (min) | | Reaction rate (pmol/cm$^2$/min) | |
|---|---|---|---|---|---|
| | | Pure PC | PC + c-Fos | Pure PC | PC + c-Fos |
| $PLA_2$ | 14 | 1.80 ± 0.10 | 1.60 ± 0.17 | 27 ± 3 | 38 ± 3 |
| | 16 | 2.40 ± 0.57 | 3.36 ± 0.82 | 5.4 ± 1 | 19 ± 4 |
| PLC | 15 | 2.47 ± 0.16 | 4.45 ± 0.49 | 45 ± 1 | 35 ± 1 |
| | 18 | 9.50 ± 1.50 | >25 | 32 ± 3 | No activity detected up to 25′ |

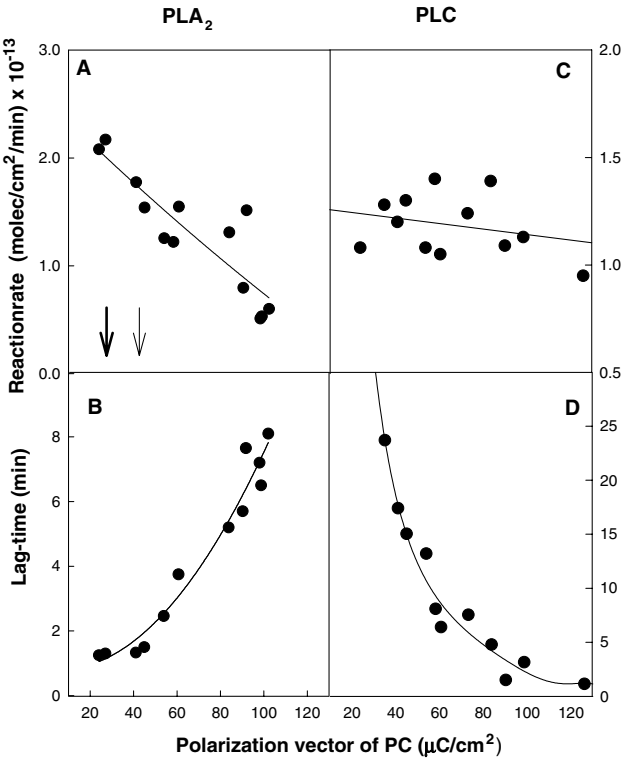


Fig. 3. Variation of $PLA_2$ (A) and PLC (C) activity and the respective change of lag time in the corresponding lower panels (B, D) as a function of the dielectric polarization vector of PC. These values (black points), re-elaborated from experiments reported in [19], were obtained in mixed films of PC with a variety of components that affect phospholipase activity in different manners due to their different interfacial polarization and interactions with PC, at the optimum surface pressure for each reaction. Lines are the best regression polynomial to the scatter plots and are shown only to indicate the general trend. The thin arrow in A indicates the magnitude of the dielectric polarization vector for PC in pure films; the thick arrow shows the same parameter for PC in mixed films with 0.08 molar fraction of c-Fos. The scales on the left correspond to $PLA_2$ and those on the right correspond to PLC.

creases and the interface becomes hyperpolarized. Note that the dielectric polarization vector of PC changes from about 40 μC/cm$^2$ in pure PC films to 27 μC/cm$^2$ in the presence of c-Fos (thin and thick arrows, respectively, in Fig. 3A), at the surface pressure at which the phospholipase activity was measured. On the other hand, the polarization of PC has little effect on the reaction rate of PLC (Fig. 3C), but the hyperpolarization of the interface greatly increases the reaction lag-time (Fig. 3D) to a magnitude in which the steady state activity can no longer be apparent. This is because the impairment for accomplishing the required precatalytic steps hinders the progress of the reaction in the time frame of our measurements of activity. Our observations that the polarization state of the interface increases when PC mixes with c-Fos (this work) can now be correlated to our previous findings showing that c-Fos increases $PLA_2$ and abolishes PLC enzymatic activity on PC [3].

### 3.3. Microscope examination of PC-c-Fos films

In order to see if any further observable features arise in mixtures of PC with c-Fos that could influence phospholipase activity (i.e., lateral domain segregation [12]), films were examined using EFM and BAM simultaneously under progressive compression. For mixed films with 6 mol% c-Fos (Fig. 4), EFM shows a rather homogeneous topography at all surface pressures. BAM images also reveal little featured surfaces, with some regions of higher reflectance, especially above 10 mN/m, similar but larger than the small reflective clusters seen in pure protein films (not shown). Above 13 mN/m BAM images show the same diffuse pattern; EPF coincides in showing a mostly homogeneous liquid-expanded phase, with occasional gray domains. Thus, microscopy coincides with the analysis of the bidimensional phase diagram in that mixed films do not reveal conspicuous domain segregation.

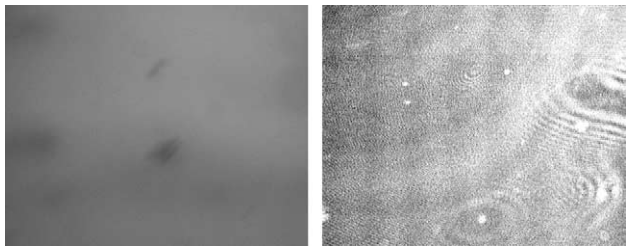

Fig. 4. Epifluorescence (left) and Brewster angle (right) images of a PC-c-Fos mixed film in the range of 10–15 mN/m. The vertical side of the BAM image corresponds to 4.8 mm. The magnification of the epifluorescence image is 10 times that of the BAM image.

## 4. Discussion

We have previously proposed that changes in substrate organization may be a possible mechanism for the modulation of phospholipase activity by c-Fos [3]. We now show that c-Fos indeed mixes with PC in a non-ideal manner, inducing marked changes of phospholipid packing and dipole potential. At mol fractions of c-Fos between 5 and 9 mol%, and surface pressures between 10 and 15 mN/m (the proportion – 8.6 mol% – and the surface pressure range over which c-Fos differentially modulates phospholipase activity [3]), the partial molecular area of PC is expanded by about 150%, as compared to protein-free films. Concomitantly, the partial surface potential per unit of molecular surface density, which is directly proportional to the overall molecular dipole moment perpendicular to the interface, indicates that the phospholipid is hyperpolarized by more than 200% in the presence of c-Fos (Fig. 2). Such changes in the molecular organization of the interface can result in the modulation of phosphohydrolytic activity that depends on lateral packing, surface electrostatics, or the phase state [8,12,22,23]. The two-dimensional phosphohydrolytic reaction is a complex catalytic process, including pre-catalytic steps such as interfacial enzyme adsorption and activation, that take place during a lag-time period preceding steady-state product formation [8,12,23]. Factors that disrupt the packing of phospholipids in a bilayer and increase their effective area should contribute to the activation of phospholipases, while a condensation of the substrate would result in reduction of phospholipase activity [7,12,24].

We previously reported that c-Fos activates phospholipase $A_2$degradation of PC above 12 mN/m [3]. We now find that it induces PC to expand at surface pressures roughly above 10 mN/m. $PLA_2$ activity, similar to sphingomyelinase [12], is favored when the substrate is in the liquid-expanded state [6]; independently, it is increased by local or externally induced

hyperpolarization (decrease of the dielectric polarization vector, see Fig. 3A) of the PC substrate [19,25,26]. Pre-catalytic activation of $PLA_2$ can remain unaffected by c-Fos as indicated by little variation of the lag-time period (Table 1) [19,26]. Thus, both the molecular expansion and hyperpolarization of PC induced by c-Fos result in the increased $PLA_2$ activity previously reported [3].

Contrary to $PLA_2$, c-Fos reduces PLC activity on the same substrate and over the same range of surface pressures [3], indicating, as previously suggested [8,12,19,26], that lipid packing is not the only mechanism operating to modulate phosphohydrolytic activity. Rather, the concerted combination of different interfacial effects concurs to regulate different phospholipases. Although lipid expansion might in principle favor PLC activity, its accessibility to the lipid interface or its pre-catalytic activation may be hindered by the presence of the protein. For phospholipases of type C, the pre-catalytic latency period has been correlated to kinetically more restricted steps such as interfacial enzyme adsorption, slow rate-limiting steps for interfacial activation, surface domain defects and induction of bilayer contacts [27,28]. We reported previously that c-Fos induces a large increase of the lag-time for PLC, which impedes progress of the reaction [3]. Fig. 3C and D shows that the presence of lipids or proteins that locally depolarize or hyperpolarize the substrate affects only slightly the steady-state rate of activity, but PC hyperpolarization induced by c-Fos impairs the occurrence of pre-catalytic steps as indicated by the marked increase of the lag-time. Thus, even if substrate degradation by PLC could eventually be allowed by surface packing, catalysis may not occur if the pre-catalytic steps have not been accomplished within an effective time period due to the modified substrate electrostatics.

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