SPECTROSCOPIC STUDIES ON THE MEMBRANE LIPIDS OF MAMMALIAN CELLS

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Summary. - \(^1\)H and \(^3\)P NMR spectroscopy and static fluorescence polarization have demonstrated to be valuable tools for studying intact membranes of mammalian cells. Phospholipid headgroups are monitored as surface probes by \(^3\)P NMR, while \(^1\)H NMR and fluorescence monitor the properties of the inner core of the membrane. The most recent studies on isolated membranes, microsomes and total cells are reviewed and discussed.

Riassunto (Proprietà spettroscopiche delle strutture lipidiche nelle membrane plasmatiche delle cellule di mammifero). - La spettroscopia di risonanza magnetica nucleare del protone e del fosforo, insieme con la tecnica dell'aneltropia di fluorescenza, si sono dimostrate tecniche molto potenti per lo studio delle proprietà strutturali delle membrane totali delle cellule di mammifero. I nuclei di fosforo delle teste polari dei foslolipidi rappresentano infatti dei probe naturali per l'analisi dell'organizzazione strutturale a livello della superficie di membrana, mentre la RMN del protone e la fluorescenza danno informazioni sugli strati interni della struttura. Nel presente articolo vengono riportati i più recenti risultati ottenuti in questo settore sulla membrane isolate e su cellule in toto.

Introduction

Many spectroscopic techniques have shown to be very powerful in elucidating the static and dynamic structures of lipids in cell membranes [1]. A large amount of data comes from studies performed on systems such as model membranes constituted by extracted lipids both alone or interacting with some selected proteins or other molecules of biological interest. However, these techniques would, in principle, give the most valuable contribution in studying intact membrane structures in isolated total membranes or even in whole unperturbed cells. Among the great body of literature we therefore selected the most recent papers dealing with this latter topic.

In this review we focussed our attention on the information available from the \(^1\)H and \(^3\)P NMR studies and its relationship to what was obtained by means of static fluorescence polarization. In fact, these two techniques have been successfully used in the study of intact membranes, particularly for cancer cells. The information of \(^3\)P NMR, provided by \(^3\)P as a surface probe, is complementary to that deduced by \(^1\)H NMR and static fluorescence polarization, usually adopted for probing the properties of the inner core of the membrane.

The results obtained hitherto on unperturbed membranes encourage to expect that this approach will lead us to a better understanding of the membrane mediated response of cells to environmental stimuli and to cell-cell interactions.

Static fluorescence and \(^1\)H and \(^3\)P NMR techniques in the membrane studies

A very powerful tool for monitoring the membrane fluidity is provided by the fluorescence polarization of hydrophobic chromophores embedded in the lipid matrix. By considering that the molecules present in the bilayer are hindered in their motion, the term "microviscosity" has been used to characterize the membrane fluidity. The steady-state fluorescence anisotropy, \(r\), (or polarization, \(p\)) has been considered as an index of mobility restriction. In fact, the fluorescence emitted from molecules in a viscous medium is partially polarized. The molecular anisotropy \(r\) and the degree of polarization \(p\) are defined as

\[
\begin{align*}
\gamma &= \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} & (1)
\end{align*}
\]

\[
\begin{align*}
p &= \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} & (1')
\end{align*}
\]

where \(I_{\parallel}\) and \(I_{\perp}\) are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to
the plane of polarization of the excitation beams. For a rotating sphere, $r$ and $p$ follow the Perrin equation [1]

$$r^0 = \frac{1}{p} \left( \frac{1}{3} \right) = 1 + 6 \frac{K_s}{\lambda}$$

(2)

where $r^0$ and $p^0$ are the limiting values for $r$ and $p$ in a very viscous medium, $K_s$ is the rate of rotation of the sphere and $\lambda$ the rate of fluorescence emission. Introducing the value of $R$ from hydrodynamic considerations we obtain [2]:

$$\frac{r^0}{r} = 1 + \frac{kT \tau}{\eta v}$$

(3)

where $T$ is the absolute temperature, $\eta$ is the viscosity of the medium, $v$ the effective volume of the fluorescent sphere, $\tau$ is the average life-time of the excited state and $k$ is the Boltzmann constant. By considering the deviation of the used probes from a sphere the usually used formula is [3, 4]:

$$\frac{r^0}{r} = 1 + \frac{kT \tau}{\eta v^0}$$

(3')

where $v$ is termed as microrviscosity of the system and is the harmonic mean of the effective viscosities opposing the in and out-plane rotations of the plane. As stressed by Shinitzky and Yuli [4], $\eta$ is the term of direct physiological relevance; nevertheless the fluorescence anisotropy $r$ (or polarization $p$) can be used as qualitative scale for fluidity, due to the direct relationship (equation 3') between $r$ and $\eta$. Particularly $r$ (or $p$) is expression of both the angular range and the rotational rate of the lipid chain motion. The first is related to the order parameter and the second to the rotational correlation time. These two contributions can be resolved only by means of time resolved fluorescence measurements. In steady-state experiments $r$ (or $p$) are indicative of mobility restriction but, hitherto, the main data of literature have been obtained by the steady-state technique.

Many natural modulators of lipid fluidity, of chemical and physical nature, have been envisaged. In the first category we recall the cholesterol percentage, the degree of unsaturation of the phospholipid acyl chains, the level of membrane proteins, the lipid composition. Physical effectors are temperature, pressure, pH membrane potential, ions.

The membrane fluidity is related not only to the physical state of the fatty acid chains, but also to the headgroup behavior. Below the phase transition temperature, when the acyl chains are in the extended all-trans conformation and are closely packed, the range of motion is small. Above the phase transition, the chains contain a number of gauche configurations, the packing of the chains is looser and the range of angular motion open to the acyl chain is increased, as the motion rate.

The presence of phosphatidylethanolamine (PE) in the bilayer determines an increase in lipid microviscosity, due to the ability of the PE headgroups to form hydrogen bonds. Also sphingomyelin is known to be an extremely rigidifying agent due to the presence of ceramide groups.

Another technique which might, in principle, provide information on the situation of the hydrophobic core of the bilayer is the H NMR. In fact, the resonances of $\text{CH}_2\alpha$ and terminal $\text{CH}_3$ of the lipid acyl chains can directly monitor the mobility properties of these groups without introducing perturbative probes. Unfortunately, due to the reduced lipid mobility, mainly consequence of interactions with cholesterol and proteins, the fatty acid chains in intact membranes are known to generate lines of about 10 kHz, even when the membranes are in the liquid-cristalline state [5-7], and the amount of knowledge on chain configuration, obtained in model systems, is usually restricted in the total membranes.

A very original approach to the study of the natural membrane structure is provided by the $3^P$ NMR methodology. In fact, the $3^P$ NMR spectra of the membranes are extremely characteristic for the different lipidic polymorphic phases and extremely sensitive to changes of phases, giving rise, in this latter case, to spectra due to the superposition of many structure spectra. This allows the contemporary presence of different structures to be detected at the same time, particularly in biological systems where alternative techniques, such as X rays, are not of a simple use.

Very extensive reviews on this subject are provided by scientists who first discovered and studied the phenomenon [8, 9]. We want to briefly remind the spectral features that characterize the different structural situations.

Natural or model membranes of phospholipids in bilayer phase, when the particles have a radius around 2,000 Å, show a spectrum (Fig. 1 a) whose broadness derives from two causes. First, the chemical shift anisotropy of the phosphorus nucleus must be considered. In fact, the $3^P$ nucleus, experimenting different orientations with respect to the external magnetic field $\mathbf{H}$, resonates at different frequencies. The extreme positions are observed when the angle between the director axes and the magnetic field is $0^\circ$ and $90^\circ$. The chemical shielding anisotropy for a generic orientation angle $\delta$ is defined with respect to the isotropic frequency $v_i$ as

$$\Delta v(\delta) = \frac{v(\delta)}{v_i} - 1.$$  

(4)

The difference between the frequencies relative to these extreme orientations yields the chemical shift anisotropy $\Delta v_{CSA}$:

$$\Delta v_{CSA} = \Delta v(0^\circ) - \Delta v(90^\circ).$$

(5)

This quantity is positive for the bilayer configuration. The shape of the spectrum of Fig. 1 a is generated by the probability distribution function $P(v)$ of
orientations, thus leading to a narrow symmetric spectrum.

Despite the interesting promises deriving from the useful information that might come from the examination of $^{31}$P NMR spectra of membranes, few studies have been performed on intact cell membranes. This fact derives mainly from the difficulty in preparing well characterized membrane systems. On the other hand, signals from membranes in intact cells are difficult to be detected, because of the presence of the very narrow and intense lines from phosphorylated metabolites. From these two reasons, the studies performed hitherto with this technique mainly deal with model membranes composed of only lipids and/or reconstituted lipid-protein systems.

**Isolated membranes and microsomes**

Functionally intact rescaled erythrocyte membranes are a suitable model of natural membranes, representing a simple system with a biochemical and structural organization very similar to that found in intact cells. Reliable and simple techniques for the preparation of erythrocyte ghosts have been known over many years [10]. The resulting system maintains many of the properties found in the plasma membrane of the intact cell. For these reasons, erythrocyte ghosts have been largely employed both for the study of the erythrocyte membrane per se and as a model of natural membranes, mainly under the effect of perturbing agents.

$^{31}$P NMR studies have clearly indicated that the lipids in different biological membranes from bacterial and eukariotic sources mostly adopt a bilayer configuration. This is true also for erythrocytes. Fig. 2 reproduces erythrocyte ghost spectra (a) and liposomes composed of their extracted lipids (b). In both cases, despite the presence of large amounts of phosphatidylethanolamine, the bilayer configuration is assumed by the membrane. On the contrary, in ether extracted ghosts and in liposomes composed of lipids from ether extracted ghosts, the lipid segregation induces the formation of phase different from the bilayer (Fig. 2 c and d). The $\Delta V_{CSA}$ is similar among all the spectra of Fig. 2 and, at 37 °C, is about 40 ppm.

This erythrocyte bilayer spectrum is very stable towards temperature and pH variations as well as towards lipid degradation [11]. However, when fusogen agents are introduced into the membrane, disruption of the bilayer structure occurs, due to extensive formation of nonbilayer phases [12-14].

A comparative study of the effects induced on the erythrocyte membrane by different lipophilic vitamins has allowed morphological modifications produced by these vitamins to be correlated with their fusion ability. Infact, vitamins A, E and K1, all producing cell fusion, also induce the formation of configurational phases other than the bilayer (Fig. 3 b, d and e). In particular, vitamin A induces the formation of isotropic and hexagonal phases; vitamin K1 produces the same effects,
but at higher doses. On the other hand, vitamin E induces the formation of only the isotropic phase even when its concentration is strongly increased. Vitamin D3 exhibits a totally different behavior: an extended aggregation phenomenon with no cell fusion is paralleled by bilayer phase stabilization (Fig. 3 c). These experimental results are in agreement with the observation that fusion is favoured by the presence of unsaturated fatty acids in the bilayer, due to the increasing portion of hydrocarbon chain in a relative liquid state [15]. In fact, the isoprenoid chain of vitamin A has the strongest effect due to the presence of cis double bonds.

Different changes are observed in the $^{31}$P NMR spectra, when the erythrocyte bilayer structure is perturbed by ionizing radiation [16]. In particular, a decrease of the chemical shift anisotropy $\Delta \gamma_{CSA}$ is observed as a function of the radiation dose. The phenomenon, accompanied by observations of chemical modifications of both cytoskeletal proteins and lipids (mainly of the PE fraction) was attributed to modified lipid-protein interactions [16].

Besides the erythrocyte membrane, among natural membranes the microsomes extracted from liver endoplasmatic reticulum have received much attention because of their intense metabolic activity, notably related to very important enzyme controlled reactions, such as oxidative desaturation of fatty acids [17], synthesis of cholesterol and triglycerides [18], glycogen breakdown [19] and protein synthesis [20]. Moreover, the procedure for microsomal membrane preparations in native conditions is well established [21]. By using the phospholipid phosphorus as a non perturbative intrinsic probe, it has been demonstrated that liver microsomal phospholipids are unique, among natural membranes, in adopting structures characterized by more isotropic motion than what was observed for phospholipids in the bilayer configuration [22]. The obvious explanation that the motional averaging, due to tumbling of the entire microsomal vesicle, can be sufficient to mediate to zero the dipolar broadening has been rejected on the basis of the persistence of an isotropic spectrum even in very viscous or dehydrated environments [22]. Moreover, it has been observed that both rabbit liver [23] and rat liver [24] microsomes display a $^{31}$P NMR spectrum characterized by a strong temperature dependence. A bilayer isotropic phase transition is in fact observed for an aliquot of the total lipids, at increasing temperature from 4 to 37 °C. Liver slices [24] and hepatocytes [25] also display a similar behavior, indicating that lipid domains of non bilayer structure characterized by high mobility are present in liver membranes. In microsomes this could be explained by a high PE concentration 20-25% of the total lipids, with respect to the PE concentration characteristic of plasma membranes.

Table 1 gives the values of steady-state fluorescence parameters of fluorescent probes (mostly DPH), imbedded in a variety of natural membranes. It has recently been suggested [26] that the steady-state anisotropy is more sensitive to the packing of the bilayer than to its
Table 1. - Fluorescence anisotropy $r$ and polarization $p$ for the DPH probe in isolated biological membranes and in normal and transformed cells

<table>
<thead>
<tr>
<th>Kind of membrane/cell</th>
<th>Temperature ($^\circ$C)</th>
<th>$r$</th>
<th>$p$</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple membranes</td>
<td>10</td>
<td>0.334</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.302</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocyte membranes</td>
<td>10</td>
<td>0.295</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.219</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
<td>10</td>
<td>0.220</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.132</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial membranes</td>
<td>10</td>
<td>0.223</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal extracts</td>
<td>15</td>
<td>0.096</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>39.5</td>
<td>0.092</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-M fibroblast</td>
<td>25</td>
<td>0.196</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Normal lymphocytes</td>
<td>25</td>
<td>0.295</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic lymphatic leukemia</td>
<td>25</td>
<td>0.277</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>25</td>
<td>0.274</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>25</td>
<td>0.305</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Lymphoma cells</td>
<td>25</td>
<td>0.217</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Neuroblastoma (N-18) a</td>
<td>37</td>
<td>0.017 $d$</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.007 $e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma (N-18) b</td>
<td>37</td>
<td>0.052 $f$</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.038 $g$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma (N-18) c</td>
<td>37</td>
<td>0.158 $d$</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.156 $e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3 A 31</td>
<td>37</td>
<td>0.110</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>3T3 d</td>
<td>37</td>
<td>0.122</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>3T3 Py6</td>
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<td>0.137</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>py6R1</td>
<td>37</td>
<td>0.100</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>SVT2</td>
<td>37</td>
<td>0.150</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>L1210</td>
<td>25</td>
<td>0.114</td>
<td>0.162</td>
<td>49</td>
</tr>
<tr>
<td>D 17</td>
<td>25</td>
<td>0.166</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Btk 21</td>
<td>25</td>
<td>0.171</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>HeLa</td>
<td>25</td>
<td>0.201</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>NDV-MDBK</td>
<td>25</td>
<td>0.247</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>N-egg</td>
<td>25</td>
<td>0.242</td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

$*$: probe 12 PS; $\ddagger$: probe pyrene; $\alpha$: probe ANS; $d$: cells in monolayer; $e$: cells in suspension.
viscosity. The change of lipid packing produced by external effectors may modulate a different cell function [27, 28]. For example, it was possible to correlate cell fusion (or aggregation) induced by lipophilic vitamins with variations of the fluorescence anisotropy of DPH probe embedded in erythrocyte membranes [13, 14, 28].

The membrane of the cancer cell

The lipid composition and, consequently, the fluidity properties of cancer cell membranes have been demonstrated to be very different from what was usually found in the correspondent normal cells [29], due to some unknown necessities for the tumoral state. Much interest is, therefore, elicited by the studies of the variations induced by the tumoral condition in the local and total membrane fluidity of cancer cells. However, one of the major difficulties in performing such studies is the availability of a method that gives specific answers to the membrane structure in the total cell or a reliable procedure for extracting unperturbed membranes. High resolution NMR has recently shown to be a powerful technique for obtaining information on unusually fluid lipid domains associated with the tumoral state.

As already stated in the previous sections, the phospholipid headgroups and fatty acid chains, present in biological membranes, produce relatively broad NMR resonances, due to the slow tumbling rate of the cells or reconstituted membranes and to the constraints on the motions imposed by the bilayer structure. From these reasons, only very broad signals can be observed by $^3$P and $^1$H NMR techniques in the isolated membranes or extracted lipids.

As stated in the NMR section, phospholipids isotropically tumbling and not in diffusive exchange with other membrane lipids can, in principle, be detected by high resolution $^1$H NMR, on the basis of the theory of motional averaging [30]. The occurrence of these conditions was first reported by Block et al. [31] and subsequently confirmed by Mountford et al. [32] on cancer cell suspensions (Fig. 4 a) and excised solid tumors (Fig. 4 b). Other reports on a high resolution lipid spectrum included not transformed cells such as fibroblasts, peripheral blood B lymphocytes and stimulated T and B lymphocytes (33-36). The $^1$H NMR spectra reveal a relatively broad methylene resonance arising from the acyl chain of lipids in a membrane structure characterized by a relatively high motional freedom. The lipid peak from tumor cells, shown in Fig. 4 a, resolved by Lorentzian-Gaussian deconvolution, reveals four resonances under the broad peak at 1.2 ppm, attributed to different lipid domains. Association of the observed peaks with the plasma membranes is demonstrated by the addition of the paramagnetic ion Gd that affects the chemical shift of the interacting species, while the same ion has been shown to bind exclusively outside the cell. Moreover, independent groups [37-39] have demonstrated that the spectra of the isolated membranes, extracted by totally different procedures, also display the methylene peaks characteristic of intact cells (Fig. 5).

By accurate analysis of the NMR relaxation properties of one of the four methylene components it has been found a correlation between the very long T2 values observed for cells and excised tumors and their capacity to metastasize [40]. Very similar $^1$H NMR features were observed in the sera from tumor bearing patients [41]. The isolation of an RNA-proteolipid complex from the same sera indicated the same structure as responsible for the high resolution proton spectra. The similarities observed in the spectral parameters, and particularly in the relaxation properties, of sera and cells, led the authors to propose a model [42], suggesting the same RNA-proteolipid complex both in sera and cell
membranes associated with a malignant disease. The mobile fatty acid chains, observed as narrow resonances, were mainly attributed to triglyceride molecules.

More evidence for the presence of similar small structures, in which the motional averaging is able to produce high resolution spectra, has come from $^{31}$P NMR of cancer cells in culture [38, 39]. In fact, the spectra from both isolated membranes and intact cells from human colon adenocarcinoma show the presence of a relatively narrow and structured signal, not related to the active metabolism and positioned at a frequency typical of a phosphodiester signal from lipids. Moreover, a signal around the same frequency is also observed in the phospholipid extracts (PCA) from the cells.

This lipid structure undergoes some thermotropic changes, at least at the headgroup level, as demonstrated by disappearance of the signal at low temperatures [38, 39]. This behavior is very similar to what observed in $^{31}$P NMR from liver slices [24].

It is somewhat surprising that lipid structures might be extracted by acid treatments and then be soluble in an aqueous environment; nevertheless, it has been shown that water soluble proteolipid complexes can be extracted by mild acid treatment from the erythrocyte membrane [43]. In the presence of phosphatidylserines, that possess unusual solubility in water and low affinity for chloroform, together with their association with proteins, was demonstrated and accounted for the stability of the complex in water.

Enzymatic degradation of the extracted structures confirms the model proposed by Wright et al. [41] on the presence of a RNA-proteolipid complex, also containing phospholipids as demonstrated by $^{31}$P NMR [38, 39], in the plasma membrane of cancer cells.

At present, there is not sufficient information about these membrane structures and their role in the tumoral membrane. The widespread of NMR spectroscopy in biomedical research encourages to think that these studies might be helpful in the cancer management.

There are debates on the meaning of fluorescence parameters for the membranes of transformed cells. In fact, variations in polarization anisotropy were observed by many authors, after transformation, as reported in Table 1. It can be deduced that the lipid fluidity, observed by means of fluorescence studies, is somewhat modified in transformed cells compared with normal cells. Some authors [44] claim that these variations are attributable to the structural and dynamical properties of the transformed membrane. Other authors [29] are more conservative in interpreting their fluorescence data, because conflicting results are often reported, and fluidity changes are not associated with tumor transformation but rather with changes in cell growth rate and density dependent cell growth. Furthermore, attachment of cells to surfaces is also known to modify the $r$ parameter for mouse neuroblastoma cells, compared with suspended cells [45].

Moreover, the different values of polarization anisotropy could be originated by different distribution of the label in the various cell membranes, due to variations in the phospholipid compositions of the cell membranes after cell transformation.

In conclusion, the fluorescence techniques, though particularly suitable for membrane studies because of their high sensitivity, do not allow general statement on the modifications induced in the plasma membrane by the tumoral transformation.

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