Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 60 (2003) 543–554
Talanta
www.elsevier.com/locate/talanta

Evidence for lipids–calcium ions interactions using fluorescent probing in paediatric nutrition admixtures

V. Hernando [a,b,*], A. Rieutord [a,b], F. Brion [b], P. Prognon [a]

[a] Faculté de Pharmacie, Laboratoire de Chimie Analytique EA 3343, 5 rue J.B. Clément, 92290 Chatenay-Malabry, France
[b] AP-HP, Hôpital Robert Debré, Service de Pharmacie et Laboratoire de Toxico-Pharmacologie, 48 Bd Sérurier, 75019 Paris, France

Received 18 July 2002; received in revised form 23 December 2002; accepted 10 January 2003

Abstract

The aim of this work was to envisage a new analytical fluorescent method to study the molecular interactions between cations and negatively charged lipid droplets contained in total parenteral nutrition (TPN) admixtures. For this purpose, two fluorescent probes were tested: 9-diethylamino-5H-benzo[α]phenoxazine-5-one, commonly named nile red (NR), and 2-(*p*-toluidinyl)-naphthalene-6-sulfonate (TNS). NR, a neutral molecule, and TNS, an anionic one, are both polarity probes. Their fluorescence emission was enhanced in an apolar environment. They were used at 1 and 2.5 μM, respectively. Results showed that scattered light was very intense in weak aqueous dilution (1/10 v v^{-1}) of fat emulsion and appeared as an experimental constraint. The sensitivity of fluorescence measurement in fat emulsion samples was constantly higher for NR than for TNS. When calcium addition occurs, as in pharmaceutical practice, a dramatic increase of fluorescence emission signal was showed for TNS, but no effect was observed for NR. As a conclusion, it was pointed out that the interactions between lipid droplets and calcium ions were likely to take place at the interface of the droplet and that TNS was a more appropriate probe than NR to prove it. Thus, fluorescent probing appeared to be a convenient new analytical tool for the investigation of lipid-cations interactions in TPN mixtures.

Keywords: Nile red; 2-(*p*-Toluidinyl)-naphthalene-6-sulfonate; Lipid emulsion; Calcium ions

1. Introduction

Intravenous lipid emulsion is a major component of total parenteral nutrition (TPN). It consists of an oil-in-water emulsion i.e., oil droplets dispersed throughout an aqueous dispersion medium with the help of an emulsifying agent [1]. The latter commonly used in parenteral fat emulsion is natural lecithin, extracted from egg yolk. Lecithin principally includes phospholipids, in which phosphatidylcholine is the major component. Phospholipids include in their molecular structure an ionised headgroup and two fatty acid chains, which confer to them both polar (hydrophilic) and apolar (hydrophobic) regions. Due to this characteristic, phospholipids pack together at the oil/water interface to form the lipid monolayer of

* Corresponding author. Tel.: +33-1-40-03-57-41; fax: +33-1-40-03-24-81.
E-mail address: vanessa.hernando@rdb.ap-hop-paris.fr (V. Hernando).

0039-9140/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0039-9140(03)00104-8

the oil droplet. Ionisation of the hydrophilic portion of the emulsifier agent confers negative surface charges to fat droplets. The charged surface then acts to repel the close approach of other similarly charged droplets and contribute to the physical stability of the emulsion [2]. Cations, introduced in the TPN mixtures, can interact with negative surface charges of lipid droplets, inducing the loss of negative surface potential of lipid droplets. The progressive disappearance of electrostatic repulsive forces eventually leads to the aggregation of lipid droplets and to the instability of TPN mixtures [1].

Stability of TPN mixtures has been widely investigated [2,3]. Classical methods, to assess it, are based on the checking of physico-chemical features of the emulsion. Among them, zeta potential measurements or particle sizing techniques are the most frequently implemented. However, they raise some technical problems. In particular, they require a very important dilution of lipid sample in water or in an electrolytic solution [4–6]. Moreover, they do not provide any information about species at the molecular scale.

In this context, it seems that fluorescent probes could be a convenient method to analyse the interactions between lipid droplets and cations, since they have been widely used to study various lipid structures, such as biological membranes [7–10] or liposomes [11,12]. Fluorescent probes exhibit spectroscopic properties, which are influenced by the modifications of the molecular environment (for example polarity, fluidity, pH, electrostatic potential...). When inserted in macromolecular structures, they can provide structural and dynamic information at a molecular scale about the sample studied [13–15].

The aim of our work was to develop a new analytical method using fluorescent probes to study molecular interactions between fat emulsion and cations, in order to get a better insight concerning instability mechanisms of TPN admixtures. For this purpose, two fluorescent polarity probes were selected: 9-diethylamino-5H-benzo[α]phenoxazine-5-one, commonly named nile red (NR), and 2-(*p*-toluidinyl)-naphthalene-6-sulfonate (TNS).

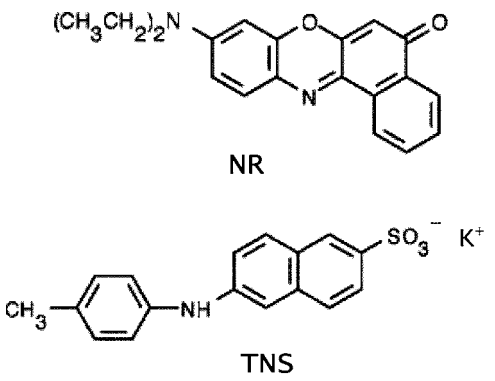


Fig. 1. Molecular structures of NR, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, and of 2-[*p*-toluidinyl]-naphthalene-6-sulfonic acid, potassium salt (TNS).

NR (Fig. 1) is a planar, heterocyclic, hydrophobic benzophenoxazone dye. It can be dissolved in a wide range of organic solvents, but negligibly in water [16]. The dye is weakly fluorescent in water and polar solvents and highly fluorescent in non-polar solvents. There is a red shift of both excitation and emission spectra upon transfer to a more polar solvent [17,18]. Fluorescence of NR is dramatically increased upon binding to lipids; NR has been used as a fluorescent stain for intracellular lipid droplets [19]. NR is photochemically stable. It exhibits a low basicity ($pK_a = 1.00 \pm 0.05$) and consequently its protonated form is only found in very acidic, polar solvents [17].

TNS (Fig. 1) is a parent compound of the well known probe 1-anilinonaphthalene-8-sulfonate (ANS). ANS and TNS are able to bind non-covalently to proteins as well as lipids. They have been used to study membrane composition and structure, and hydrophobic regions of proteins [20]. Because the pK_a of sulfonate group lies between 0 and 1, pH variations in the range from 2 to 11 have no influence on the ANS fluorescence [21]. Emission fluorescence of ANS pronouncedly depends on the nature of the solvent, especially on its polarity, whereas excitation spectra is almost independent of polarity. The same considerations can be applied to TNS which is chemically and spectroscopically very similar to ANS [15].

In our study, we will first optimise experimental conditions and evaluate whether NR and TNS are

appropriate for our pharmaceutical problems. Then, interactions between fat emulsion and calcium ions will be studied.

2. Material and methods

2.1. Apparatus

Steady state fluorescence measurements were performed on a LS50B Perkin Elmer® (Les Ulis, France) luminescence spectrometer, driven by the FL WINLAB Perkin Elmer® software. Hellma® cuvettes, with a 1-cm light path cell, made of quartz Suprasil from Roucaire (Les Ulis, France) were used. pH measurements were done with a 691 pH meter Methrom® provided by Roucaire.

2.2. Reagents

2-[*p*-Toluidinyl]-naphthalene-6-sulfonic acid, potassium salt (TNS) was purchased from Sigma-Aldrich® (Saint-Quentin Fallavier, France). 9-Diethylamino-5H-benzo[α]phenoxazine-5-one, NR, was produced by Molecular Probes® (Eugene, USA) and obtained from Interchim® (Montluçon, France). Methanol was from Carlo Erba® (Val de Reuil, France). Deionised water was obtained from a millipore® MilliQ® (Saint-Quentin en Yvelines, France). All solvents used were of analytical grade and spectroscopically tested before experiments. Intra-venous fat emulsion, Ivelip® 20% from Clintec Parenteral S.A. Laboratory (Montargis, France), was chosen for our experiments. Intra-venous aqueous solutions of calcium gluconate 10% ($m\,v^{-1}$) from Aguettant Laboratory (Lyon, France) was also used.

2.3. Methods

2.3.1. Preparation of probe solutions and fat emulsion samples

10^{-3} M methanolic fluorescent probes stock solutions were prepared and kept for 3 months at $-20\,^{\circ}C$. They were then diluted at 10^{-4} and 10^{-5} M in methanol, as required.

Fat emulsion samples were daily prepared by diluting Ivelip® 20% at 1/10 $v\,v^{-1}$ in deionised water. Daily dilution was made to prevent possible chemical or physical degradation of lipid emulsion.

Commercial calcium solution were used pure or half-diluted with deionised water.

2.3.2. Fluorescence measurements

All experiments were performed in triplicate, at room temperature ($20\pm2\,^{\circ}C$). All samples were prepared in glass tubes. The order of introduction of the reagents was always as follow: fat emulsion sample, then calcium solution or corresponding volume of water, and eventually probe solution or corresponding volume of methanol. For each experiment, an appropriate blank was prepared. Its signal was collected (F_0) and systematically subtracted from the fluorescence intensity (F) of the sample. Fluorescence response $F/[P]$ ($AU \times M^{-1}$) was determined as the ratio of fluorescence intensity F in arbitrary units (AU) divided by the probe concentration [P]. Excitation–emission slits were adjusted depending on the experimental conditions.

2.3.3. Light scattering measurements

To study light scattering in fat emulsion samples and in water, total fluorescence spectra were systematically collected in both media. Pseudo-three-dimensional plot of fluorescence emission spectra (isomeric projection) were obtained by recording successive emission spectra ($n=30$) from 350 to 700 nm, at stepped increments of excitation wavelengths fixed at 10 nm. The excitation–emission slits were set at 10/10 nm.

2.3.4. Linearity of the fluorescence probe emission

The linearity of the fluorescence signal as a function of probe concentration was checked in fat emulsion samples. Probe concentrations tested were 0.1, 0.5, 1, 2.5, 5 and 10 μM. Each curve was repeated three times. All samples were made by mixing 850 μl of fat emulsion sample, 100 μl of aqueous calcium solution or water and 50 μl of methanolic probe solution or methanol. Each sample contained the same proportion ($v\,v^{-1}$) of fat emulsion sample, water and methanol. Final

dilution of Ivelip® emulsion in samples was equal to 1/11.7 v v^{-1}.

A least square regression (LSR) was used to verify the existence of linear relationship between fluorescence intensity and probe concentration. One way analysis of variance was performed to check the significance of the slope. To assess if the intercept of the linear regression equation was or not significantly different from zero, a Student test was performed. The level of significance was always set at $p < 0.05$.

Quantification limit (QL) was calculated as follow:

$$QL = \frac{10\sigma_b}{a}$$

σ_b is the standard deviation of the intercept of the linear regression equation. a is the slope of the linear regression equation.

Statistical treatment of data was performed using EXCEL® software.

2.3.5. Study of lipids–calcium ions interactions

2.3.5.1. Effect of calcium on fluorescent probe emission linearity. The modification of the fluorescence signal as a function of probe concentration was investigated in fat emulsion samples, in the presence of calcium ions (22 mM). Probe concentrations tested were 0.1, 0.5, 1, 2.5, 5 and 10 μM. The same experimental conditions, as detailed in Section 2.3.4, were applied.

2.3.5.2. Effect of calcium on fluorescent probe emission intensity. The variation of fluorescence signal (F) as a function of calcium concentration was studied in fat emulsion sample for TNS and NR. In the same way, the signal (F_0) in a blank sample of identical composition, but in the absence of fluorescent probe, was also collected. Calcium concentrations studied were 1.1, 2.2, 3.3, 4.4, 6.6, 8.8, 13.2, 17.6 and 22 mM.

3. Results and discussion

3.1. Optimisation of experimental conditions

3.1.1. The fluorescence properties of the tested probes in fat emulsion

For our study, two polarity probes were a priori selected: NR and TNS. To check if both probes exhibit characteristics of polarity probes, of interest for our problem, excitation and emission fluorescence spectra of NR and TNS were recorded in fat emulsion (Ivelip® 1/10 v v^{-1}) and compared to methanol and water. Methanol (dielectric constant $\varepsilon = 32.7$) was chosen as a model for a relatively weak polarity medium, and water ($\varepsilon = 78.4$) for a strong polar one. The corresponding NR and TNS spectra obtained are respectively shown in Figs. 2 and 3. Wavelengths of maximum excitation and emission, as well as fluorescence response measured in different media are presented in Table 1 for both compounds.

NR and TNS showed fluorescence properties of polarity probes: a drastic increase of fluorescence intensity when dissolved in a non-polar medium (methanol or fat emulsion). Their emission maximum was also blue-shifted with decreasing polarity, as shown in Table 1. For NR, we observed a blue-shifted excitation maximum when decreasing polarity, whereas excitation maximum of TNS did not significantly change, suggesting that energy level of the ground state S_0 remains unaffected for TNS in these three studied environments. Spectral behaviour observed for NR and TNS are related to general and specific solvent effects.

The general solvent effects are exhibited by all dyes. They are caused by the interactions of dipoles of the probe with dipoles of neighbouring solvent molecules. The higher the polarity of the solvent, the higher the interaction and thus the higher the energy lost in solvation effects. Therefore, the fluorescence spectrum exhibits a gradual spectral red shift with increasing polarity of the solvent, accompanied by a decreasing of quantum yield.

The specific solvent effect is an additional property of some dyes. It depends upon the precise chemical structures of the solvent and the fluorophore. Specific interactions include hydrogen

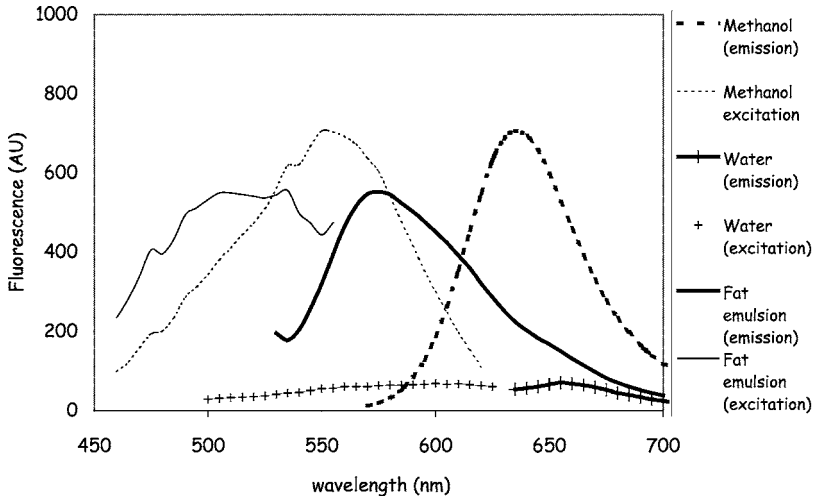


Fig. 2. Excitation and emission fluorescence spectra of NR in methanol, water and fat emulsion samples ([NR] = 10^{-6} M in methanol, 10^{-5} M in water, 10^{-6} M in fat emulsion; excitation–emission slits = 10/5 nm in methanol, 10/10 nm in water, 10/10 nm in fat emulsion).

bonding, acid–base chemistry and formation of charge transfer complexes [22].

TNS, like its analogous ANS, only exhibits general solvent effects [15]. On the contrary, NR exhibits general and specific solvent effects like a twisted intramolecular charge transfer [23], leading to a more complex spectral behaviour than TNS.

For TNS, replacement of water by less polar solvents, such as methanol or fat emulsion, resulted in the appearance of structural details in excitation spectrum i.e. the resolution of vibrational energy levels of the ground state. This phenomenon can be explained by decreasing solvent–fluorophore interactions in an apolar

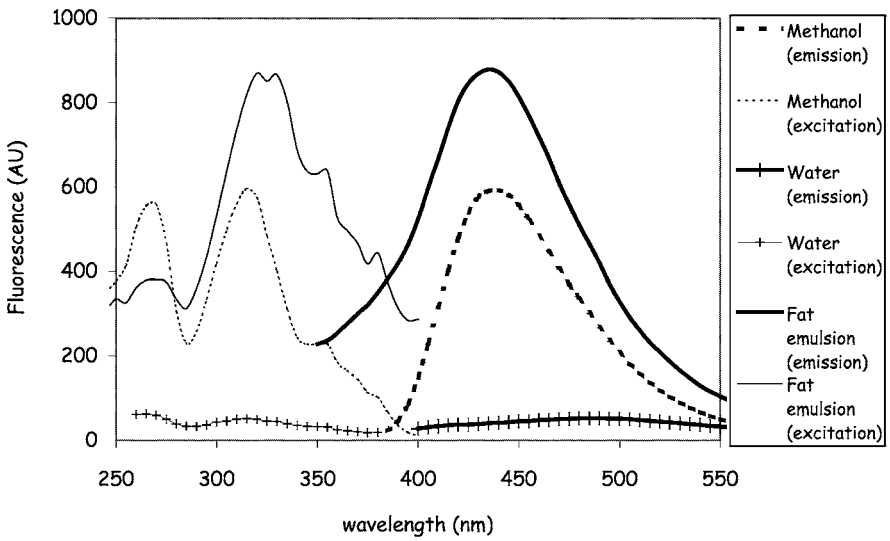


Fig. 3. Excitation and emission fluorescence spectra of TNS in methanol, water and fat emulsion samples ([TNS] = 10^{-6} M in methanol, 10^{-5} M in water, 10^{-5} M in fat emulsion; excitation–emission slits = 10/5 nm in methanol, 10/10 nm in water, 10/10 nm in fat emulsion).

Table 1
Fluorescence properties of NR and TNS in water, methanol and fat emulsion

Solvent	NR			TNS		
	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence response ($AU \times M^{-1}$) (mean ± S.D.)	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence response ($AU \times M^{-1}$) (mean ± S.D.)
Water	608	659	16 ± 3[a]	317	490	1 ± 0[a]
Methanol	550	636	632 ± 31[a]	316	438	612 ± 23[a]
Fat emulsion	534	575	563 ± 33[b]	320	439	85 ± 4[b]

[a] Excitation–emission slits set at 10/5 nm.
[b] Excitation–emission slits set at 10/10 nm.

environment [22]. Emission spectrum of TNS is devoid of structure unlike excitation one. Such deviation from the mirror image rule of fluorescence usually indicates a different geometric arrangement of moieties in the excited state as compared to the ground state [22]. NR excitation spectrum also showed spectral structure differences with varying polarity.

Both NR and TNS emitted a fluorescence signal in fat emulsion samples, suggesting that they were likely to be partitioned into the lipid phase of the emulsion rather than in the aqueous one. However, fluorescence enhancement was greater for NR than for TNS (Table 1). This feature may be related to the ionisation state of both probes. To verify this hypothesis, pH measurements were performed in fat emulsion. Results are shown in Table 2. At nearly neutral pH values, as measured in all fat emulsion samples, NR is a non-charged molecule, whereas TNS is an anionic one. Anionic state of TNS could lead to the establishment of electrostatic repulsive forces with negative surface charges of lipid droplets, needing to increase the probe concentration used (10 μM for TNS vs 1 μM for NR) to favour its partition into the lipid phase of the emulsion. Because of its charge, TNS is supposed to be a probe of the oil/water interface in the lipid droplets [24]. In contrast, the neutral NR partitions preferentially into the lipid phase. NR is considered as a probe of the phospholipid monolayer in lipid droplets [25]. Thus, the use of both probes to study lipids–cations interactions in TPN mixtures might permit us to determine the location of the interaction within the emulsion (oil/water interface or phospholipid layer).

Table 2
pH measurements in fat emulsion samples

Fat emulsion sample	pH (mean ± S.D.)
Ivelip® 1/10 ($v\ v^{-1}$)	6.68 ± 0.08
Ivelip® 1/10 ($v\ v^{-1}$) + NR ($[NR] = 10^{-5}$ M)	6.69 ± 0.05
Ivelip® 1/10 ($v\ v^{-1}$) + TNS ($[TNS] = 10^{-5}$ M)	6.73 ± 0.03
Ivelip® 1/10 ($v\ v^{-1}$) + calcium gluconate ($[Ca^{2+}] = 10$ mM)	5.93 ± 0.00

3.1.2. The light scattering problem in fat emulsion samples

As fat emulsion samples are very turbid, a high degree of scattered light can not be avoided. The importance of scattering in measurement of fluorescence is great, particularly in turbid solutions, since a certain proportion of the scattered light is always detected and interferes with the fluorescence spectra. To investigate this point, a pseudo-three-dimensional plot of fluorescence emission spectra, in the absence of fluorescent dye, was systemically recorded. Typical records obtained in water (Fig. 4a) and in fat emulsion sample (Fig. 4b) are presented in Fig. 4.

Scattering phenomena due to the reemission of exciting light with no energy transfer are clearly detected at $\lambda_{exc} = \lambda_{em}$. Reemission by solvent molecules in water led to Rayleigh scattering [a]. In fat emulsion, Tyndall scattering [d] was present together with Rayleigh scattering giving a more intense interfering signal than in water. Tyndall scattering was due to the reemission by lipid particles in fat emulsion [15].

Scattering phenomena arising from reemission of exciting light following an energy transfer in the form of vibrational energy was also noteworthy. In water, solvent molecules were the cause of Raman scattering [b]. In fat emulsion sample, lipid droplets of different size, led to an intense scattered light [e]. According to Rayleigh's law [26], intensity of light scattering from particles in suspension is a function of the angle of observation (θ). It increases proportionally with the square size of particles and with decreasing exciting wavelength. In our samples, this feature has to be taken into account, particularly at low emission wavelengths. Therefore, for our measurements in fat emulsion, NR will present the advantage on TNS of high excitation and emission wavelengths (Table 1), limiting light scattering interferences.

Second order diffraction ([c] and [f]) is a consequence of the instrumentation used (optical system).

Light scattering in fat emulsion was also evaluated for other Ivelip® dilutions i.e., 1/100, 1/1000 and 1/10 000 v v^{-1}. Results (data not shown) surprisingly pointed out that scattering light was more important in Ivelip® 1/100 v v^{-1} and in Ivelip® 1/1000 v v^{-1} than in Ivelip® 1/10 v v^{-1}. The most appropriate dilution for fluorescence measurements was 1/10 000 v v^{-1}, because leading to the lowest light scattering. However, we did not choose this dilution for our further experiments because it was not close enough to the composition of paediatric nutrition admixtures prepared in hospital. In these admixtures the dilution of Ivelip® ranges between 1/7.4 and 1/11.8 v v^{-1}. Consequently, dilution for Ivelip® was definitively set up at 1/10 v v^{-1}, despite the above cited relatively high light scattering.

3.1.3. Dynamic of the tested probes response

Before studying fat emulsion-cations interactions, the optimal added concentration of the probe was determined. This concentration should be as low as possible, to induce minimal perturbations on the studied structure. Obviously, it should be at least equal to the limit of quantification of the probe to ensure that the fluorescence intensity collected is high enough to be easily distinguished from the background signal. It should be also included in the linearity range of the probe fluorescence signal.

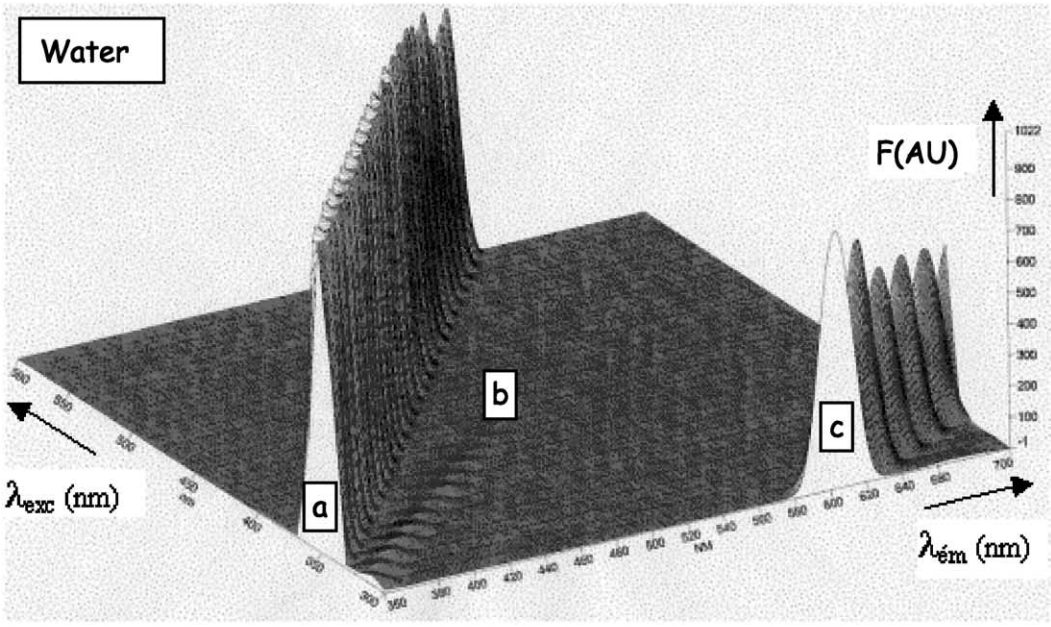
To determine the optimal concentration of each probe, the linearity of fluorescence intensity, as a function of probe concentration, was established in fat emulsion samples. Results obtained for NR and TNS are showed in Table 3.

All LSR equations were characterised by a slope and an intercept significantly different from zero ($p < 0.05$).

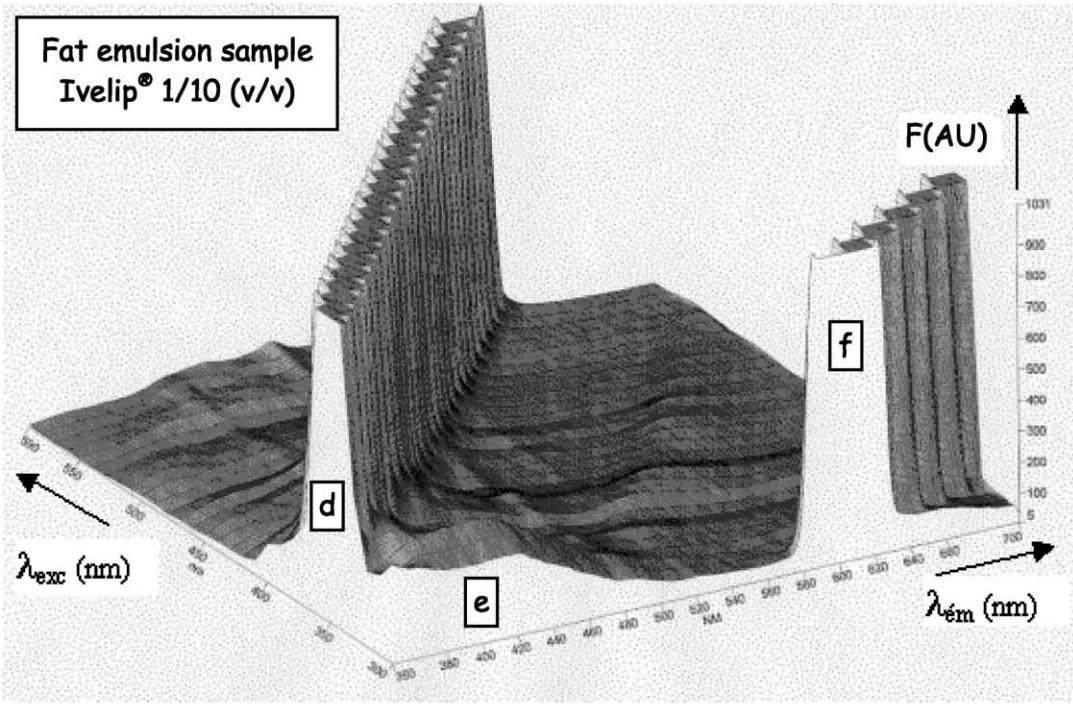
For NR, optimal concentration was set at 0.5 μM. The absence of linearity observed at higher NR concentrations might be explained by an inner filter effect.

No inner filter effect was detected for TNS and 2.5 μM was chosen as the optimal concentration.

Comparing results for both probes, it was noteworthy that the slope of the LSR equation was higher for NR than for TNS ($p < 0.05$), suggesting that sensitivity of the measurement was greater for NR than for TNS. The interaction between the probe and the lipid droplets was probably stronger for NR than for TNS, according to the partition of both probes between lipid and aqueous phases as previously discussed.



(a)



(b)

Fig. 4. Pseudo-three-dimensional plot of fluorescence in water (4a) and fat emulsion sample (4b) in the absence of fluorescence dye.

Table 3
Linearity of NR and TNS fluorescence emission as a function of probe concentration in fat emulsion sample (Ivelip® 1/10 v v^{-1}) in the absence and in the presence of calcium gluconate (22 mM)

Calcium salt	LSR equation	Correlation coefficient (r)	Linearity range (μM)	Quantification limit (μM)
NR[a]				
−	$y = 191x + 13$	0.998	0.1–1	0.16
+	$y = 212x + 10$	0.997	0.1–1	0.17
TNS[b]				
−	$y = 18x + 182$	0.986	0.1–10	1.98
+	$y = 92x + 187$	0.971	0.1–1	0.55

[a] Excitation–emission slits set at 10/6 nm, $\lambda_{exc}/\lambda_{em} = 505/575$ nm.
[b] Excitation–emission slits set at 10/7 nm, $\lambda_{exc}/\lambda_{em} = 320/439$ nm.

Lastly, the intercept of LSR equation was greater for TNS than for NR ($p < 0.05$). The scattering light, more intense in TNS emission area than in NR's one, could explain this observation.

3.2. Study of lipids–calcium ions interactions

To study lipid-cations interactions, calcium was chosen as a model. First of all, because it is a main inorganic species introduced in TPN composition. Secondly, calcium as a divalent cation, is a more destabilising ion than a monovalent one. Finally, calcium has been already used to study TPN mixtures stability by the mean of zeta potential measurements [27]. Among all calcium salts, calcium gluconate was selected as the one of the most frequently used in practice.

3.2.1. Effect of calcium on fluorescent probe dynamic range

The influence of calcium gluconate addition (22 mM) on the linearity of the monitored fluorescence signal of the probes was established in fat emulsion samples. Results are reported in Table 3.

First of all, the absence of fluorescence enhancement for both probes by the mere addition of calcium was checked in water (data not shown).

Second, for NR, the addition of calcium led to no significant modification of the slope or of the intercept of calibration curve ($p > 0.05$). Then, the interaction of uncharged NR with lipid droplets appeared to be independent from the presence of calcium ions in the medium. Consequently, fluorescence emission of NR would probably provide no information about the lipids-cations interactions.

Concerning TNS, the intercept of the linear equation was not modified after the addition of calcium but the slope of the curve was significantly increased ($p < 0.05$). The presence of calcium in the environment might lead to a higher interaction between TNS and lipid droplets. In fact, calcium could neutralise negative surface potential of lipid droplets and decrease electrostatic repulsive forces between lipid droplets and the anionic probe. The enhancement of the slope is likely to be proportional to the increase of the number of TNS molecules interacting with lipid surface and consequently to the affinity of the studied cation for lipid droplets. Such a behaviour has been already described for ANS, a close compound of TNS, in the presence of biological membranes or liposomes [21]. At this stage, TNS seems to be a potential interesting probe to investigate the interactions between lipid droplets and cations, unlike NR.

3.2.2. Effect of calcium on fluorescent probe emission intensity

The modification of fluorescence intensity for TNS and NR was studied as a function of calcium concentration. The calcium concentration tested ranged from 0 to 22 mM. In the paediatric parenteral mixtures, prepared in our hospital, the calcium concentration range between 2.5 and 7.5 mM and total divalent cations concentration between 4.6 and 13.7 mM. Therefore, therapeutic calcium and cations concentrations have been

studied in our experiment. The signal was also collected for a blank, free of spiked fluorescent probe, exactly in the same experimental conditions that for the probe samples. The results obtained are presented in Fig. 5.

Despite the absence of fluorescent dye, a modification of the signal was measured in both blank samples (Fig. 5b). The signal collected showed an increase of about 25% for a calcium concentration ranging between 4.4 and 8.8 mM. This latter could be attributed to the variations of light scattering under the influence of the calcium addition to fat emulsion samples. As previously said, calcium ions may lead to some aggregation of lipid droplets in

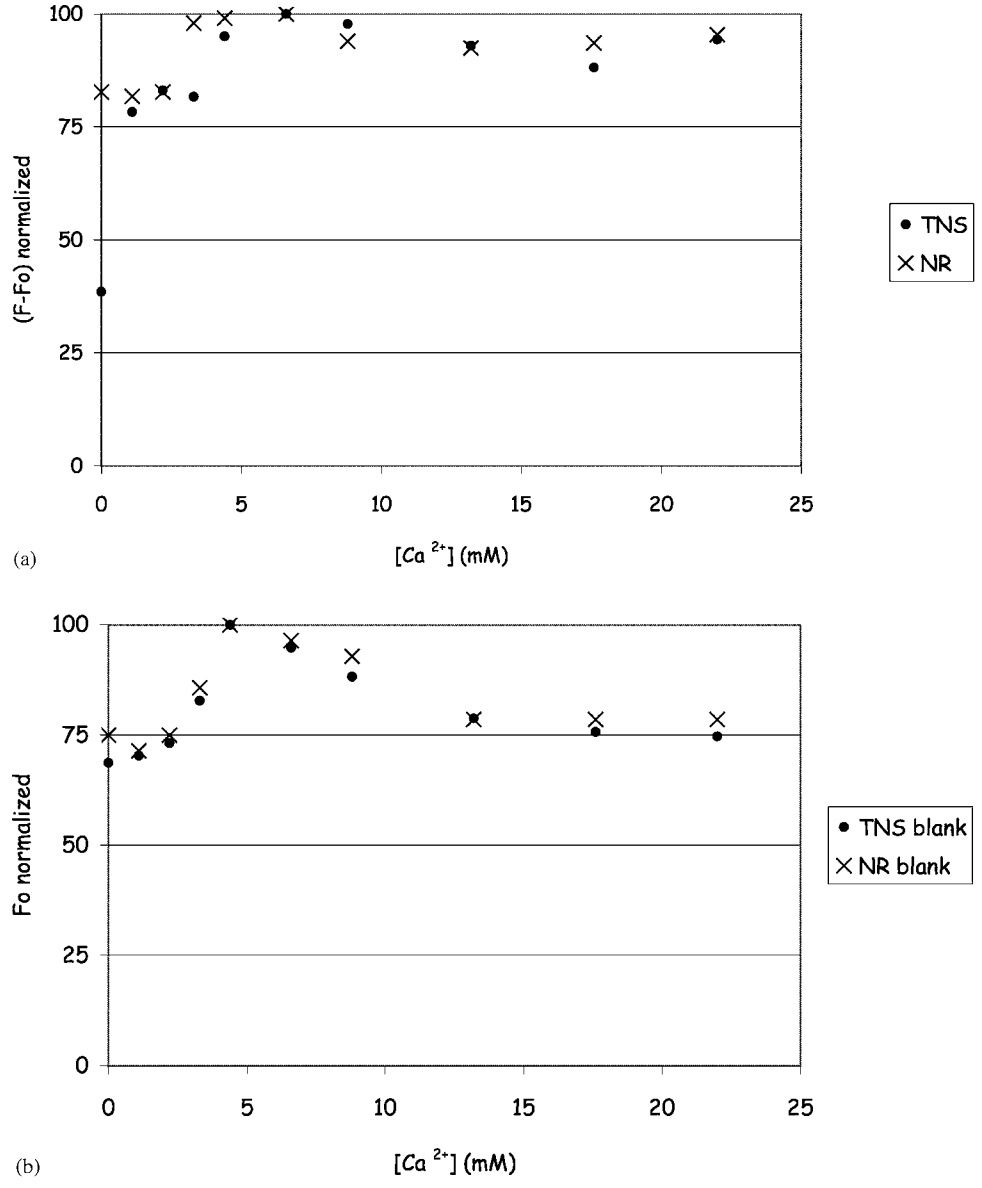


Fig. 5. Fluorescence intensity for TNS (2.5 μM) and NR (1 μM) in fat emulsion samples as a function of calcium concentration (5a); signal in the absence of fluorescent probe in the same experimental conditions (5b) i.e., for TNS, $\lambda_{exc}/\lambda_{em} = 320/439$ nm, exc/em slits = 10/10 nm; for NR, $\lambda_{exc}/\lambda_{em} = 505/575$ nm, exc/em slits = 10/6 nm.

fat emulsion. Owing to this possible aggregation, lipid particles of greater diameter are generated. As quoted before, intensity of scattering light to particles in suspension is increased with the square size of the latter.

In the presence of fluorescent dye (Fig. 5a), the curve profile was different for NR and TNS. NR showed a similar profile as the one collected in the NR blank sample. By contrast, TNS exhibited a dramatic increase of fluorescence intensity as soon as 1.1 mM of calcium was added. The fluorescence was increased by about a factor 2. Then, a second enhancement was detected from a calcium concentration of 5.5 mM, similarly to the TNS blank sample. The intense fluorescence enhancement observed for TNS, at low calcium concentrations, may be related to an increased interaction between TNS molecules and fat droplets under the influence of calcium ions, as previously explained. At calcium concentrations below 4.4 mM, TNS fluorescence increased according to a hyperbolic function of calcium concentration. The graph of the double reciprocal plot of fluorescence increment against cation concentration could allow the calculations of the binding constant between calcium and lipid droplets, even in a such heterogeneous system. Attempts to determine this binding constant are currently under investigation.

4. Conclusion

These preliminary results demonstrated that fluorescent probes are an attractive tool to study interactions between fat emulsion and cations. TNS appeared to be a more appropriate probe than NR to investigate them. The differences in the response of both probes, correlated with their respective location in the lipid droplets, pointed out that the interactions between lipid droplets and calcium ions were likely to take place at the surface of the droplet, without any modification of the polarity in the phospholipid layer. In a near future, TNS fluorescence measurements could provide an estimation of the lipids–calcium binding constant.

Thus, fluorescent probes may offer many advantages in the study of lipids-cations interactions in TPN mixtures. They are used at a low concentration (μM), inducing minimal perturbations in the studied structure. They allow to get some information about interactions at a molecular scale, as well as a qualitative and maybe a quantitative investigation. Measurements are performed directly on fat emulsion sample, at a dilution corresponding to the pharmaceutical practice. However, important light scattering has to be taken into account. The selection of the most favourable experimental conditions, like the use of a fluorescent probe with a high emission maximum, could limit this scattering problem. At least, attempts to correlate this technique to a reference method, as zeta potential measurement, should be implemented.

The ultimate aim of studying lipids-cations interactions is to get a better insight concerning instability mechanisms of TPN admixtures, in order to favour their compounding by hospital pharmacy departments.

References

[1] R. Brown, R.A. Quercia, R. Sigman, J. Parenter. Enter. Nutr. 10 (1986) 650.
[2] C. Washington, Int. J. Pharm. 66 (1990) 1.
[3] M.J. Barnett, A.G. Cosslett, J.R. Duffield, D.A. Evans, S.B. Hall, D.R. Williams, Drug Safety 5 (Suppl. 1) (1990) 101.
[4] J.M. Lestreit, A. Morali, F. Lorieul, I. May, Nutr. Clin. Métabol 11 (1997) 191.
[5] H. Komatsu, A. Kitajima, S. Okada, Chem. Pharm. Bull. 43 (1995) 1412.
[6] R.H. Müller, S. Heinemann, Clin. Nutr. 11 (1992) 223.
[7] L.D. Bergelson, J.G. Molotkovsky, Y.M. Manevich, Chem. Phys. Lipids 37 (1985) 165.
[8] R.F. Epand, R. Kraayenhof, G.J. Sterk, H.W. Sang, R.M. Epand, Biochim. Biophys. Acta 1284 (1996) 191.
[9] R.M. Epand, R. Kraayenhof, Chem. Phys. Lipids 101 (1999) 57.
[10] A. Akahori, Z. Jozwiak, T. Gabryelak, R. Gondko, Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 123 (1999) 209.
[11] V. Borenstain, Y. Barenholz, Chem. Phys. Lipids 64 (1993) 117.
[12] R.C.C. New, Liposomes: a practical approach, first ed., Oxford University Press, Oxford, 1990.
[13] B. Valeur, Fluorescent probes for evaluation of local physical and structural parameters, in: S. Schulman

(Ed.), Molecular Luminescence Spectroscopy, Part 3, Wiley, New York, 1998, pp. 25–84.
[14] R.P. Haugland, Handbook of Fluorescent Probes and Research Products, eighth ed., MTZ Spence, Eugene, 2001.
[15] J. Slavik, Fluorescent Probes in Cellular and Molecular Biology, first ed., CRC Press, Boca Raton, 1994.
[16] P. Greenspan, S.D. Fowler, J. Lipid Res. 26 (1985) 781.
[17] J.F. Deye, T.A. Berger, Anal. Chem. 62 (1990) 615.
[18] D.L. Sackett, J. Wolff, Anal. Biochem. 167 (1987) 228.
[19] E. Bonilla, A. Prelle, J. Histochem. Cytochem. 35 (1987) 619.
[20] W.O. McClure, G.M. Edelman, Biochemistry 5 (1966) 1908.
[21] J. Slavik, Biochim. Biophys. Acta 694 (1982) 1.
[22] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, first ed., Plenum Press, New York, 1983.
[23] N. Ghoneim, Spectrochim. Acta Part A 56 (2000) 1003.
[24] K. Kachel, E. Asuncion-Punzalan, E. London, Biochim. Biophys. Acta 1374 (1998) 63.
[25] M.M.G. Krishna, N. Periasamy, J. Fluoresc. 8 (1998) 81.
[26] J.P. Péré, Techniques spectroscopiques en biochimie analytique, first ed., Centre régional de documentation pédagogique d'Aquitaine, Bordeaux, 1999.
[27] C. Washington, A. Athersuch, D.J. Kynoch, Int. J. Pharm. 64 (1990) 217.