EFFECT OF pH ON THE CONTROL RELEASE OF MICROENCAPSULATED DYE IN LECITHIN LIPOSOMES – PART II

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ABSTRACT

The objective of our work has been the microencapsulation of dyes with lecithin from soybean, with the formation of liposomes, as a substitute for synthetic auxiliaries so as to improve the quality of the effluent.

Current scenarios promote the disintegration and leakage of the liposomes, such as, changes in temperature, pH and the use of surfactants. Since dyeing process is a mix of all these parameters, we pretended to study each one separately.

Changes in pH at constant temperature induce a release of dye similar with changes in temperature. In acid conditions we found a very fast initial dye release which doesn't occur in basic conditions.

Using carboxyfluorescein, as a pH fluorescence probe, we concluded that the liposome membrane doesn't protect the liposome interior from changes on the external pH.

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I. INTRODUCTION

In recent years liposomes have been examined as a way of delivering dyestuffs to textiles in a cost-effective and environmentally sensitive way (1).

Current scenarios promote the disintegration and leakage of the liposomes, such as, changes in temperature, acidity and the use of surfactants. Since the dyeing process is a mix of all these parameters, we pretended to study each parameter separately. In the first part of this paper temperature and surfactant effect on the control release of the dye, were examined. It was found that temperature and surfactant induce a release of the encapsulated dye resulting in rhodamine dilution and consequently alterations in the dimerization/binding equilibrium.

The aim of the present work was to examine the effect of pH on the release of the encapsulated dye on lecithin liposomes, using the some procedure as in part I, i.e., encapsulating a xanthene dye, rhodamine 6G in lecithin vesicles, as trapped markers for monitoring vesicle integrity.

It was also important to study the effect of the external pH on the internal pH of the liposome. The technique used for the direct measurement of internal pH takes in account the pH-dependent shift of emission spectra of fluorescence. Fluorescein and many of its derivatives exhibit multiple, pH-dependent ionic equilibrium (2,3). However the high leakage rate of fluorescein from cells makes it very difficult to quantify intracellular pH because the decrease in the cell's fluorescence due to dye leakage cannot be easily distinguished from that due to acidification. The use of carboxyfluorescein for intracellular pH measurements addresses this problem (4). This probe offers excellent time resolution, a very high sensitivity to small changes in internal pH and the ability to make continuous recordings on small samples (5,6).

II. MATERIALS AND METHODS

Materials

Commercial soybean lecithin containing 22% phosphatidylcholine, 20% phosphatidylethanolamine, 14% phosphatidylinositol and 10% phytoglycolipides was supplied by Stern (USA). Rhodamine 6G was obtained from Merck and carboxyfluorescein from Molecular Probes. Soybean lecithin and fluorescent probes were used without further purification.

Liposomes preparation with Rhodamine 6G

Vesicles were formed by hydrating the lipid film with an aqueous solution (containing a buffer solution (pH=5.5) and 30 mM of rhodamine 6G and sonication for 10 minutes at 20ºC and 60W. All dye, which was not included inside the vesicles, was removed by gel filtration. This

preparation resulted in a solution of "microcuvettes" that contained a concentration of dye equal to the concentration of the original solution in which the vesicles were formed. The overall concentration of the rhodamine 6G in this solution, is calculated using a dilution factor of 3/8 from the gel filtration chromatography and an encapsulation fraction determined from UV-VIS absorption (Shimadzu – UV-3101PC) of 7%. The size of the liposomes was about 0.8 μm, measured with a Mastersizer S (Malvern Instruments).

Fluorescence measurements

In order to study the effect of the pH on the release of the dye, liposomes marked with rhodamine were submitted to different pH, and fluorescence intensity was measured with a spectrofluorometer (Spex Fluorolog 212) during time. Rhodamine emission was detected by excitation at 470nm.

Liposomes preparation with carboxyfluorescein

Vesicles were formed by hydrating the lipid film with an aqueous solution (containing a buffer solution at pH=7) and $2x10^{-6}$M of carboxyfluorescein and sonication for 10 minutes at 20ºC and 60W. All dye, which was not included inside the vesicles, was removed by gel filtration.

III. RESULTS AND DISCUSSION

As we can see (figure 1A), there is an increase in fluorescence emission with increasing time and a blue shift. We know that at pH 7 at a constant temperature of 20ºC the liposomes keep the encapsulated dye for long periods of time (7). From these results we concluded that the increase of pH affect the permeability of the membrane during time. Figure 1 A shows the fitted spectra at pH=12 using the procedure developed in part I of this work (8). In brief we considered three distinct environments for rhodamine in vesicles: the aqueous phases (intern and extern) and two interfacial region (the internal and external membrane – water interfaces). In each subphase rhodamine can dimerize with a consequent drastic reduction in fluorescent quantum yield. The distribution of rhodamine molecules in the various environments is governed by a binding equilibrium of rhodamine with lipid molecules. The various equilibrium equations were defined using appropriate local concentrations. Conversion factors for bulk to each subphase concentrations were defined and calculated using the known or estimated structural characteristics of the lecithin liposomes (8). The spectral factors of rhodamine in each medium were fitted as a sum of three gaussian functions for the internal and external interfaces, while, for the aqueous phases, the experimental ones were used. Care was taken to avoid covariation between maximum of rhodamine fluorescence intensity in each medium and its local

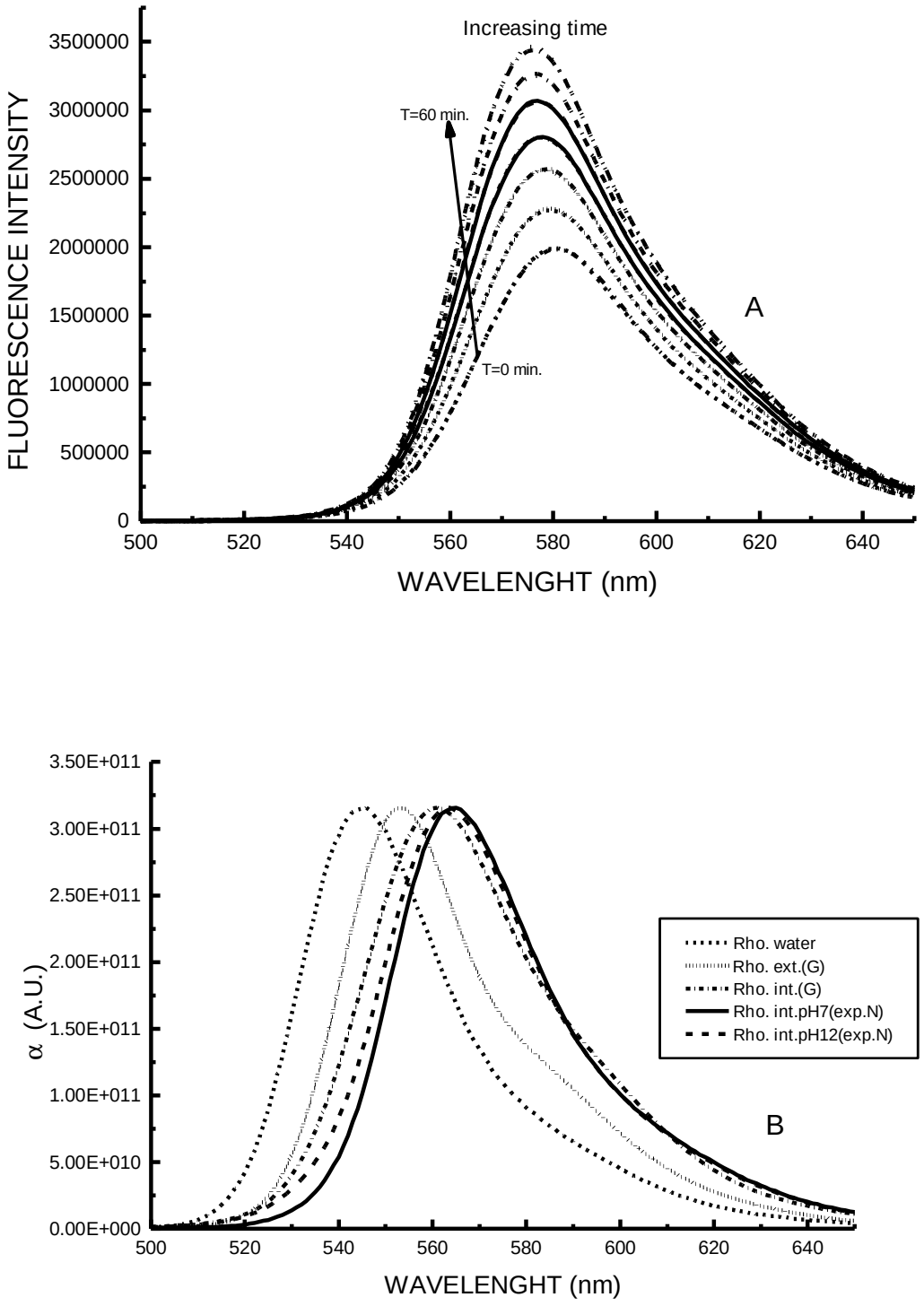

Figure 1 - Fitted and experimental fluorescence spectra of rhodamine 6G encapsulated in soybean lecithin liposomes with an external pH=12 at same temperature and at different times; B: fitted parameters, where G correspond to gaussian spectral factors and exp.N the experimental normalised ones.

concentration, by imposing a maximum variation of 2% in rhodamine maximum fluorescent intensity among the three different environments. In figure 1B the spectral factors obtained from the fitting procedure are compared with rhodamine spectra in the interior of liposomes at pH 7 and pH 12. At this point we could not tell whether the interior of the liposomes remained at pH 7 or changed to pH 12. From these results we cannot conclude whether the membrane protects the interior from alcalinization.

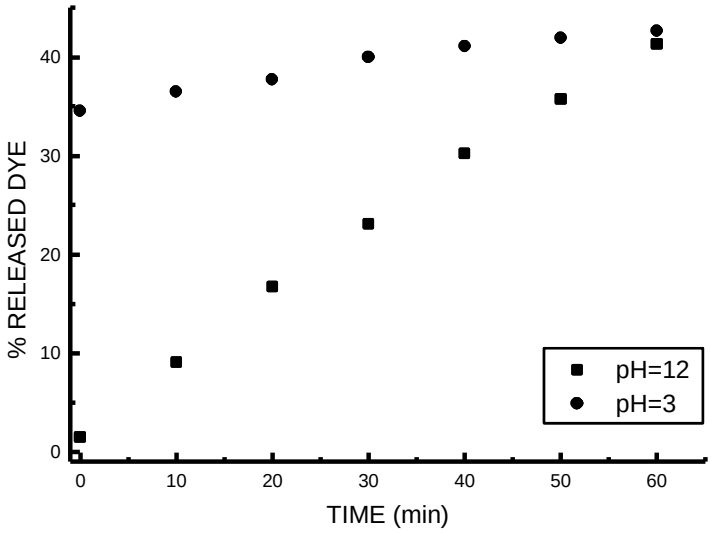

Figure 2 – Percentage of released dye from liposomes at pH 3 and pH 12.

From figure 2, at pH 12 we concluded that the % of dye release during 60 min. is about 40%. Additionally we obtained a lower dimerization constant (1.09 M^{-1}) which explains the observed much higher fluorescence intensity than the one obtained at pH 7.

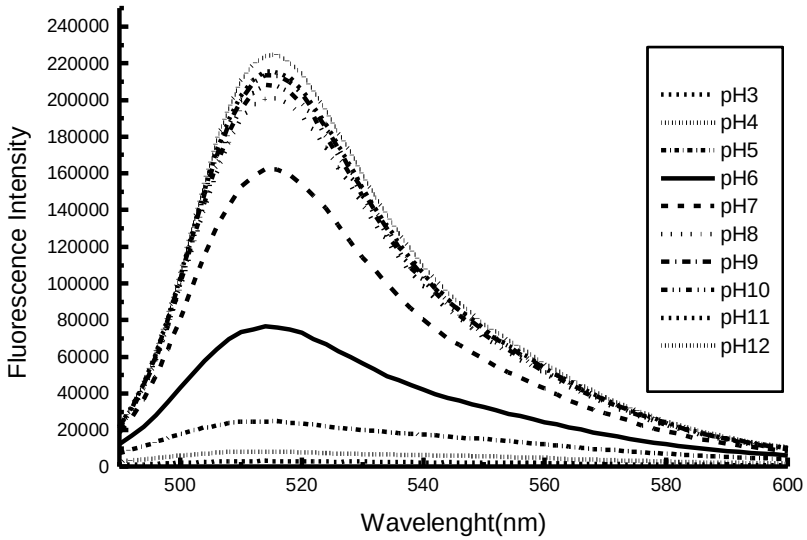

Figure 3 – pH-dependent emission spectra of carboxyfluorescein excited at 480 nm.

At pH 3 using the same fitting procedure, we obtained a very fast initial dye release followed by a smooth increase, and a dimerization constant similar to the one obtained at pH 12 (1.07 M^{-1}). Curiously, the fitted spectra at this pH, are similar to the one in the water, nevertheless we cannot exclude the possibility of partition between water and the interfaces.

In order to see if the membrane protects or not the interior of the liposome from pH changes, we decided to perform some leakage studies using carboxyfluorescein.

Figure 3 shows the pH-dependent emission spectra of carboxyfluorescein excited at 480 nm.

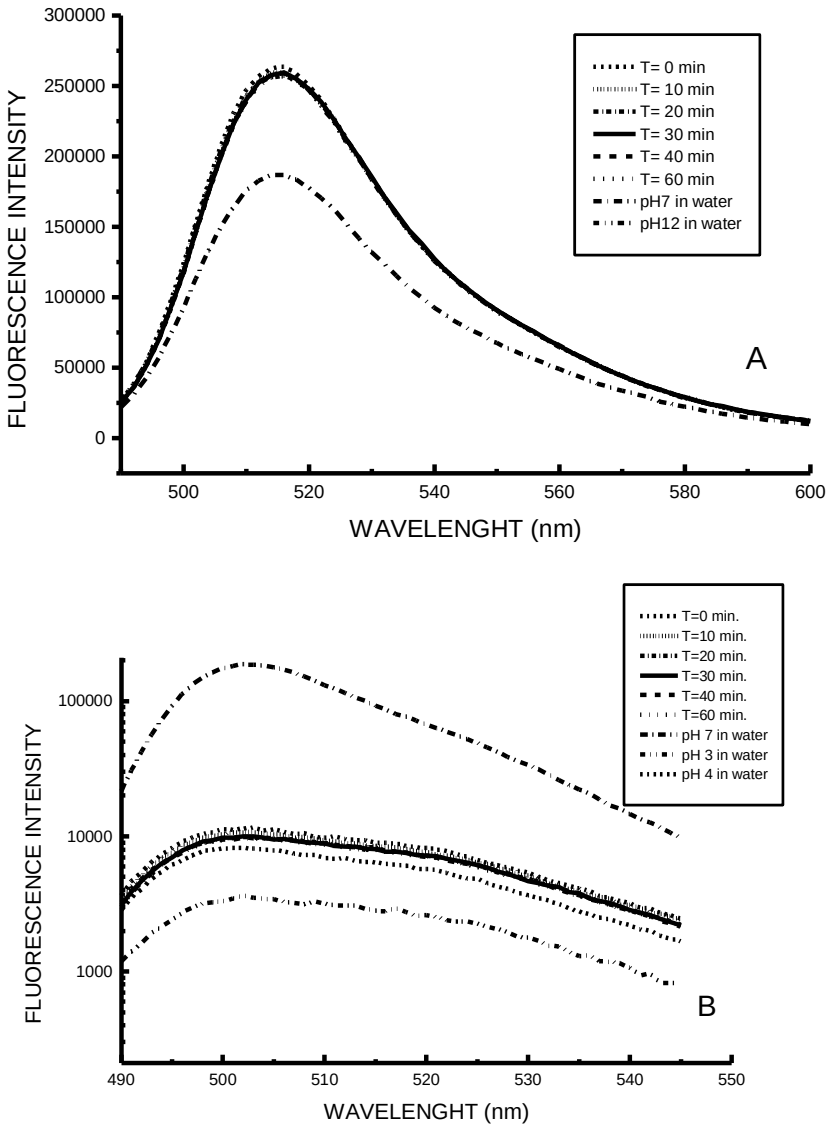

Figure 4 – Fluorescence spectra of: encapsulated carboxyfluorescein in soybean lecithin liposomes with an external pH 12 during time; carboxyfluorescein in water at pH 7 and pH 12. B: Fluorescence spectra of: encapsulated carboxyfluorescein in soybean lecithin liposomes with an external pH=3 during time; carboxyfluorescein in water at pH 7, pH 4, and pH 3.

Figure 4A shows the spectra of carboxyfluorescein in water at pH 12 and 7 at concentration of $2x10^{-6}$ M. At pH 7 the fluorescence intensity is significantly lower than the one observed from pH 12. When we changed the external pH, of the liposomes with encapsulated carboxyfluorescein (internal pH=7), to pH12 by adding an alkaline solution, we noticed that the internal pH rapidly (in less than 10 min.) approaches the external one (figure 4A).

Figure 4B shows the same study when the external pH is 3. The fluorescence intensity is significantly lower than the one observed from pH 7. Upon acidification of liposomes to pH 3 we saw a very rapid (in less than 10 min.) change in the internal pH to a value of 4 followed by a slow decrease towards the external one. Interestingly this sudden change (pH 7 to pH 4) corresponds to the very fast initial dye release observed from the previous results obtained with the acidification of encapsulated rhodamine, but in basification experiments, the same sudden pH change (pH 7 to pH 12) does not lead to an equivalent, fast release. These results can be interpreted as an indication that upon acidification, a fraction of liposomes population are disrupted and that the liposomes are resistant to basification although they are permeable to OH^- ions.

IV. CONCLUSION

pH induces a release of the encapsulated dye resulting in rhodamine dilution and consequently alterations in the dimerization/binding equilibrium.

Changes in pH at constant temperature induce a release of dye similar as the one obtained with changes in temperature. In acid conditions we found a very fast initial dye release which doesn't occur in basic conditions.

Using carboxyfluoresceine, as a pH fluorescence probe, we concluded that the liposome membrane doesn't protect the liposome interior from changes on the external pH although in acid conditions there is a residual protection effect.

REFERENCES

1. Gomes, J. I. N. R., and Baptista, A. L. F., 2001, Textile Research Journal 71 (2), 153

2. Sjoback R, Nygren J, Kubista M., 1995, Spectrochim Acta A 51, 7

3. Martin MM, Lindqvist L. ,1975, J Luminescence 10, 381

4. Buchsbaum, Thomas J.A, Zimniak ,R.N., Racker E., 1979, Biochemistry 18, 2210-2218

5. Rink, T.J.; Tsien, R.Y.; Pozzan, T., 1982, J.Cell Biol., 95, 189

6. De Clerck LS, Bridts CH, Mertens AM, Moens MM, Stevens WJ., 1994. J Immunol Methods 172, 115-124

7. Coutinho, P.J.G.; Castanheira, E.M.S., unpublished results.

8. Baptista, A.L.F.; Coutinho, P.J.G.; Real Oliveira, M.E.C.D.; Gomes, J.I.N.R, 2003, Journal of Liposome Research, to publish