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NEW APPROACH TO STUDYING LIPOSOMES: STAINING FOR VISUALIZATION AND DETERMINATION OF TRAPPING EFFICIENCY BY SPECTROPHOTOMETRY USING NEUTRAL RED

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A new rapid, simple and cost effective experimental approach to studying liposomes is proposed. Neutral red (NR) – a pH indicator and a weak base with $pK_a = 6.7$, which is red while ionized in acidic media but yellow while non-ionized in alkaline media, and is used in vital staining – was selected as a model of lipophilic and hydrophilic drugs. NR accumulates in biological membranes and liposomes in ionized form, therefore giving sharp contrast with the surrounding media of liposomes and intracellular organelles. This renders them visible in a light microscope (LM). Staining with NR has been studied using pH differences between the internal and external media of liposomes. Liposomes with differing internal (pH 5) and external (pH 9) media offer the most suitable object for such staining. The influence of differential pH on the liposome trapping efficiency (LTE) has been evaluated by spectrophotometry at a wavelength of 533 nm. The percentage LTE increased from 24 to 87% for the pH value outside (external medium) varying in the range 5 – 9. Correlations between CBC (contrast between liposomal compartments visualized by LM) and EBC (exchange between liposomal compartments) and LTE were mathematically analyzed. Significant correlations within a 95% confidence interval were observed between EBC and CBC ($r^2 = 0.93$, $p < 0.05$) and between LTE and CBC ($r^2 = 0.93$, $p < 0.05$). No significant correlation was observed between EBC and LTE with a 95% confidence interval (EBC – LTE, $r^2 = 0.66$, $p < 0.05$).

In recent decade, liposomes have become increasingly popular as vehicles for systemic delivery of drugs, enzymes, and genetic materials [1 – 5]. In addition to low intrinsic toxicity, liposomes offer protection to drugs from degradation in the circulation. Liposomes accumulate in the tumor cells (enhanced permeation and retention effect) [6], which makes them a promising delivery system for anticancer drugs. Liposomes accumulating in the reticuloendothelial system (RES) appear to be a promising vehicle to improve the therapeutic index of anti-HIV drugs such as zidovudine (AZT). However, the entrapment efficiency of AZT in liposomes was found to be low and AZT exhibited leakage from liposomes [7]. The pharmacokinetic profile of an entrapped drug is determined by the physicochemical properties of liposomes. Weak bases like doxorubicin and vincristine, which coexist in aqueous solutions in neutral and charged forms, have been successfully loaded into preformed liposomes by means of a pH gradient technique [8]. Specific functions of liposomes are directly related to pH [9, 10].

Various methods have been developed for the preparation of liposomes, the most traditional method of liposome formulation being the thin-film hydration method [11]. This method involves dissolution of lipids and a lipophilic drug in a volatile solvent, evaporation of the solvent to obtain a thin film, and hydration of the dry thin film with an aqueous vehicle to obtain a crude liposome formulation. Besides lipid composition, the method of preparation also affects the structure and characteristics of liposomes [1]. In particular, it has been shown that pH of a phospholipid solution during liposome preparation can influence the final size of liposomes and their structure [12]. In recent years, much work has been done to study liposomes, evaluate an appropriate lipid formula, and prepare size and load liposomes [13]. All systems were characterized for shape, lamellarity, particle size, and percentage liposome trapping efficiency (LTE) by transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), laser diffraction, and ultracentrifugation or dialysis methods [14].

Neutral Red (NR) is an indicator (pH 6.8 red, 8.0 yellow) for use in histology, super-vital staining, and biological staining (study of the Golgi apparatus). This dye changes from red to yellow when pH ranges within 6.0 – 8.0 [1]. In this study, staining with NR has been investigated using pH differences between the internal and external media of liposomes for their visualization by light microscopy (LM) and for the LTE determination by spectrophotometry.

Experimental Part

Materials. *L*-α-phosphatidylcholine (PC) (MW, 760.09) was purchased from Avanti Polar Lipid (Alabaster, AL); cholesterol (95%) was purchased from Aldrich Chemicals (Milwaukee, USA); normal saline (0.9%,

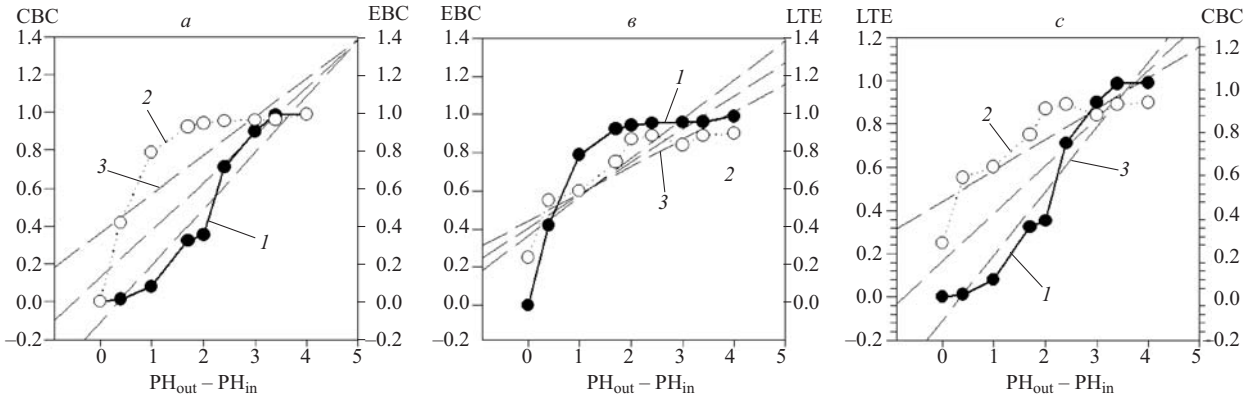

Fig. 1. Correlations between CBC, EBC and LTE with a 95% confidence interval for pH_{in} 5 and pH_{out} 5 – 9: *a*) CBC (*1*); EBC (*2*); $r^2 = 0.93$ (*3*); *b*) EBC (*1*); LTE (*2*); $r^2 = 0.66$ (*3*); *c*) CBC (*1*); LTE (*2*); $r^2 = 0.93$ (*3*)

W/v), was from Hyan Co (Iran). All reagents used were analytical or high performance liquid chromatography (HPLC) grade.

Liposome preparations. The liposomes were prepared by the reverse phase evaporation method [15]. Lipid compounds with a formulation consisting of lecithin and cholesterol in a 2 : 2 molar ratio were prepared by dissolving the lipid in a chloroform – ethanol mixture in a volume ratio of 2 to 1.5 ml. Then, the chloroform – ethanol mixture was removed by evaporation under reduced pressure (8 – 100 mbar) for about 16 h, the dried lipids were resuspended in 40 mM phosphate-buffered saline/140 mM NaCl solution at a final total lipid concentration of 3 mM, and the vesicles were maintained at 60°C for 60 min with subsequent shaking.

The buffer solution was varied depending on the type of experiments; pH was adjusted with acetic acid to 5 or with 1.0 M sodium hydroxide to 9. NR (0.01 mg/ml) is mixed with buffer (pH 5) and completely dissolved on stirring. The NR to be encapsulated in the liposomes was added to the buffer solution at a 9 : 1 ratio before evaporation of the organic solvent. Subsequently, the liposomes prepared in the presence of dye were "washed" to remove the dye from solution in which the liposomes were suspended. The washing procedure consisted of multiple cycles of centrifugation and subsequent resuspension of the pellet in dye-free buffer solution.

Table 1

Percentage of Protonated and Unprotonated NR in the External and Internal Media of Liposomes, CBC, EBC, and LTE Values

pH_{in}	pH_{out}	$\%P_{in}$	$\%P_{out}$	$\%UP_{in}$	$\%UP_{out}$	CBC	EBC	LTE
5	5	98.04	98.04	1.95	1.95	0.000	0.000	0.25
5	5.4	98.04	95.23	1.95	4.77	0.114	0.419	0.55
5	6	98.04	83.36	1.95	16.6	0.080	0.789	0.6
5	6.7	98.04	50	1.95	50	0.324	0.924	0.75
5	7	98.04	33.44	1.95	66.5	0.354	0.943	0.87
5	7.4	98.04	16.6	1.95	83.36	0.710	0.954	0.75
5	8	98.04	4.78	1.95	95.2	0.900	0.959	0.84
5	8.4	98.04	1.56	1.95	98.43	0.986	0.961	0.89
5	9	98.04	0.49	1.95	99.5	0.990	0.990	0.90

Liposome visualization by LM. A differential coloration, resulting from differences in pH between the inner and outer regions of liposomes, can bring about a sharp contrast that facilitates visualization by LM. Photograph of the stained liposomes were obtained using Leitz camera with a magnification of 40 × 3.5 × 4.

LTE determination. Liposome trapping efficiency (LTE) was evaluaded by determining the non-trapped amount using the ultracentrifugation technique. First, vesicle preparations were kept overnight at 4°C and ultracentrifuged (Pacisa FPS10). Liposome preparations (0.5 ml) to which 4.5 ml of normal saline was added, were centrifuged for 2 h at 40,000 rpm to separate free NR (in the supernatant) from that trapped in liposomes. Free NR was assayed by spectrophotometry in the supernatant. The percentage NR trapping was calculated as $LTE = (Q_t - Q_s)/Q_t$, where Q_t is the added (theoretical) NR amount and Q_s is the amount determined from NR absorbance detected by spectrophotometry in the supernatant. The NR absorption was measured at 533 nm, using various NR concentrations for calibration.

Statistical analysis. The data ware processed using Student's *t*-test and the analysis of variance (ANOVA) with $P < 0.05$ as a minimal level of significance [16]. The significance of differences between groups (different formulations) was tested using the nonparametric Ruskal – Wallis test. Results were expressed as the mean value ± standard error for $n = 5$ independent samples. All computations were performed on a computer using SPSS software.

Results

In the present study, the percentage of molecular and ionized forms of NR were calculated using the Henderson – Hasselbach formula $pK_a - pH = \log[P/UP]$, where *P* and *UP* are the percentage protonated and unprotonated NR fractions, respectively (Table. 1). Then, using a simple formula, the red stain contrast between compartments (CBC) representing the internal and external medium of liposomes was calculated as $CBC = (P_{in} - P_{out})/P_{total}$, where P_{in}, P_{out}, and P_{total} are the percentage contents of protonated NR inside an outside liposomes, and the total

protonated NR content, respectively. Table 1 presents these ratios for liposomes with the constant pH in 5 inside and variable pH out 5 – 9 outside. For example, the CBC for liposomes with the internal and external pH of 5 and 7.4, respectively, is 0.7 and that for liposomes with the internal and external pH of 5 and 9, respectively, is 0.98.

In addition, the exchange between compartments (EBC) was calculated as $EBC = (UP_{out} - UP_{in})/UP_{total}$, where UP_{in}, UP_{out}, and UP_{total} are the percentage content of unprotonated NR inside an outside liposomes, and the total unprotonated NR content, respectively. For example, the EBC ratio for liposomes with the internal and external pH of 5 and 7.4, respectively, is 0.95 and that for liposomes with the internal and external pH of 5 and 9, respectively, is 0.96 (Table 1).

According to experimental data obtained using the ultracentrifugation – spectrophotometry method for determining the level of NR trapping, the LTE value increased from 0.25 to 0.9 when the external pH was varied in the range 5 – 9 (Table. 1).

The CBC, EBC and LTE values (Table 1) were analyzed with respect to mutual correlations (Fig. 1). Significant correlations at a 95% confidence interval were observed between EBC and CBC ($r^2 = 0.93$, $p < 0.05$) and between LTE and CBC ($r^2 = 0.93$, $p < 0.05$). No significant correlation at a 95% confidence interval was found only between EBC and LTE (EBC – LTE, $r^2 = 0.66$, $p < 0.05$).

Discussion

NR is a weak alkaline compound (base) ($pK_a = 6.7$) and for $pH = pK_a$ it has a non-ionized/ionized ratio of 1 : 1. NR is yellow in the molecular form that is able to penetrate biological membranes such as cell membranes and the membranes of the organelles (e.g., vacuoles and Golgi apparatus) and artificial membrane (liposomes). The ionized form of the stain is aggregated both inside and outside the membranes and thus the ionized NR encompassed by the internal medium of liposomes cannot be exchanged between liposomes, while the molecular form can enter the biological membranes. In the acidic environment, NR is ionized inside a liposome and trapped there, while the molecular fraction of NR that enters the internal medium of liposomes remains in the molecular form and can be exchanged. The unionized NR, entering a liposome from the external medium, becomes 98.04% ionized because of the acidic pH (pH 5) while 1.95 % of it remains unionized, and can escape from the liposome.

The samples were prepared in a reservoir on a microscope slide with approximately 10 μl of solution, with liposome size ranging from approximately 0.5 to 10 μm (average, 4 μm) in diameter (Fig. 2). For the initial vesicle characterization, liposomes were examined by LM. In all cases, the spherical vesicles were predominating (Fig. 2). Some vesicles were aggregated over the entire vision field (this fact has been corroborated by other authors [17]) because of the effect of cholesterol as stabilizing agent in the lipid bilayer.

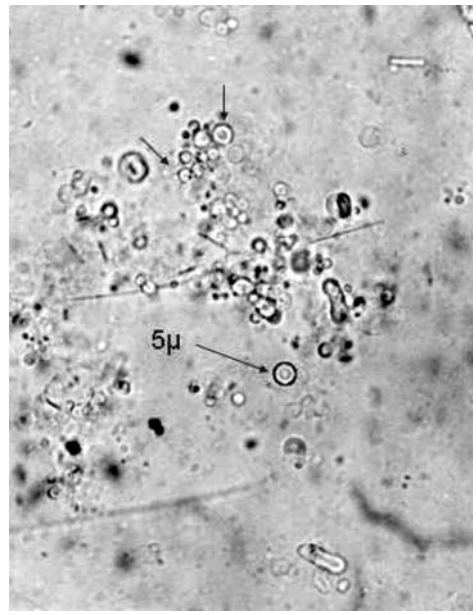

Fig. 2. A microphotograph of liposomes with internal medium pH 5 and external medium pH 9 (40 × 3.5 × 4 magnification) in a sample obtained using the reverse phase evaporation method with dimensions ranging approximately from 0.5 to 10 μm (average, 4 μm).

Liposomes become increasingly popular as vehicles for systemic delivery of drugs, enzymes, and genetic material, etc. Various methods are available for the preparation and study of liposomes. Many of these methods are neither simple nor cost effective. The purpose of this study was to develop a new experimental approach to studying liposomes. In this context, staining with NR has been investigated using the pH difference between the internal and external media of liposome for their visualization by LM and determination of the trapping efficiency of liposomes by spectrophotometry. Liposomes with differing internal (pH 5) and external (pH 5 – 9) media offer the most suitable object for such staining. The LTE was shown to increase from 0.25 to 0.9 for the pH of external media ranging from 5 to 9.

We propose to use liposomes stained with NR as a marker for the delivery of a specific drug to a tissue or an organ and the drug degradation in circulation. In this respect, various methods of liposome formulation could be compared to gain advantage of the proposed method. This aspect can be the focus of future investigations.

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НОВЫЙ ПОДХОД К ИССЛЕДОВАНИЮ ЛИПОСОМ: ОКРАШИВАНИЕ НЕЙТРАЛЬНЫМ КРАСНЫМ ДЛЯ ВИЗУАЛИЗАЦИИ В ОПТИЧЕСКОМ МИКРОСКОПЕ И СПЕКТРОФОТОМЕТРИЧЕСКОГО ОПРЕДЕЛЕНИЯ ЭФФЕКТИВНОСТИ СВЯЗЫВАНИЯ СОЕДИНЕНИЙ

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Предложен новый быстрый, простой и экономичный подход к исследованию липосом с использованием нейтрального красного (НК) красителя, являющегося pH-индикатором и слабым основанием с pK_a = 6,7. НК имеет красный цвет в ионизированном состоянии (в кислой среде), но становится желтым в неионизированном состоянии (в щелочной среде), что делает его пригодным для витального окрашивания. В данной работе НК был использован как модель липофильного и гидрофильного биологически активного соединения. Накапливаясь в ионизированном состоянии в биологических мембранах и липосомах, НК делает их видимыми в результате резкого контраста с окружающей средой, содержащей неионизированные молекулы красителя. Распределение НК *in vitro* между внутренней (pH 5) и внешней (pH 5 – 9) средой липосом и влияние разности pH на связывающую способность липосом (ССЛ) исследовано с помощью спектрофотометрии (по поглощению при 533 нм). При изменении pH внешней среды в пределах 5 – 9, ССЛ увеличивается от 24 до 87%. Корреляции между ССЛ и разницей концентраций НК в ионизированном и неионизированном состоянии внутри и снаружи липосом при различных pH исследованы с использованием данных оптической микроскопии и спектрофотометрии с окрашиванием НК.