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LipHosomes: Reporters for Ligand/Anti-Ligand Assays Based On pH Readout

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Supporting information for this article is given via a link at the end of the document.

Abstract: This work reports about a new class of liposomes (LipHosomes) designed to induce a change of pH upon releasing their content. pH-readout reports on the number of LipHosomes in the specimen. LipHosomes were prepared by entrapping NaOH or bicarbonate buffer in the intravesicular compartment. The liposomes suspension was purified from untrapped compounds and brought to pH 7.0. The pH gradient between intra- and extra-liposomal compartments is maintained because the phospholipidic membrane works as a semipermeable membrane, without diffusion of ions across the membrane. The release of the liposomal content triggers a quantifiable variation of the pH of the medium. This feature has been harnessed in analytical assays based on ligand/anti-ligand molecular recognition by exploiting the biotin-streptavidin binding scheme. A pH difference of 0.2 units was observed upon the release of the payload from biotinylated LipHosomes bound to streptavidinated plates. The test showed an excellent sensitivity being able to reveal a concentration of bound LipHosomes in the sub-pM range.

Introduction

Liposomes have been known since the middle of the last century, yet the research areas in which they find applications are still constantly growing.^[1] According to their size, liposomes can be classified as Small Unilamellar Vesicles (SUV, diameter 20 – 100 nm), Large Unilamellar Vesicles (LUV, diameter > 100 nm), and Giant Unilamellar Vesicles (GUV, diameter > 1 μ m).^[2] Up to now, SUVs and LUVs have been studied and exploited in the biomedical field to a larger extent rather than GUVs, having found important applications either *in vivo* (e.g. drug delivery)^[3] or *in vitro* assays, where they act as reporter signal amplifiers.^[4] Over the last 50 years, numerous and increasingly refined methods have been developed for the *in vitro* detection of diagnostic markers in biologic fluids, typically based on the antibody/antigen recognition (immunoassays).^[5] Among them, those based on the use of solid supports, on which the target molecule adheres or binds to in a specific way, display a very high efficiency. Enzyme-based immunoassays for various analytes have been subjected to intense scrutiny.^[6] One of the most applied technique for biomolecular analysis is the enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA-based methods consist of using chromatic variations induced by the antigen/antibody molecular recognition. For the detection of the antigen of interest, they require the use of a proper analytical tool, often represented by a spectrophotometer or a fluorimeter. The sensitivity of the method

falls in the pM range.^[7] Although good, the sensitivity is often challenged as many diagnostically relevant analytes are present in biological fluids at even lower concentration. Therefore, it appears necessary to push further down the sensitivity threshold. Other items that are considered relevant deal either with the need to reduce the analysis' costs and to access to quicker and reliable responses. Magnetic immunoassays^[8] and radio immunoassays^[9] are variants of the ELISA method. Although they are potentially more sensitive than ELISA, the detection technology is definitively more expensive than the spectrophotometric readout.

A possible strategy for performing liposome-based immunoassays consists of replacing the enzyme by specific probes encapsulated into the liposome cavity that can be detected by colorimetric,^[10] fluorimetric,^[11] chemiluminometric,^[12] photothermic^[13] or electrochemical methods.^[14] The choice of the detection method is typically made on the basis of the equipment available in the laboratory. Specific antigens or antibodies can be introduced on the liposome surface by exploiting different routes.^[15] The procedures are well established and can be applied to a wide range of antigens and antibodies.^[16]

The herein reported work reports about the development of an innovative class of liposomes that can be quantified in a given specimen by a simple pH readout. The proposed method is based on the use of liposomal vesicles whose payload is represented by solutions characterized by a pH value different from the one of the medium in which they are suspended. We call these systems with the name of LipHosomes. LipHosomes are liposomes capable of maintaining a pH gradient between the intra and extra media of the vesicles. Upon the release of the payload from the LipHosomes, a significant change in the pH of the medium occurs, that can be easily measured with a conventional pH-meter.

The herein reported results show how the proposed method is competitive in terms of the achievable sensitivity in respect to the currently available methods, with the great advantage of using a well-established, simple, fast responding, low cost detection technique as a laboratory pH-meter. Although different methods and strategies by using liposomes have been numerous reported for the bioassays on the basis of various signal-generation principles,^[10-14] there is no report focusing on liposome-based pH readout in the bioassays until now. Moreover, the great advantage of the proposed method over existing ones relies in the reduction of the analysis costs. In fact, the vast majority of the existing method make use of the reaction between enzymes and substrate to induce a variation of either absorbance or pH in the solution. The proposed method doesn't need of enzymes as amplification strategy, instead it makes use of

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bicarbonate or NaOH to induce a change in pH once these molecules are released from liposomes. The use of these very cheap reagents with respect to enzyme is supposed to drastically reduce the costs of the analysis kit. In addition, the reaction between enzyme and substrate is an additional step that cause the elongation of the analysis time and reduce the in time stability of the response. In the present work, as to provide a proof of concept of the method, the analysis scheme and its sensitivity has been tested making advantage of the well-established binding couple biotin/streptavidin but this analysis set-up can be applied to a wide range of diagnostic biomarkers present in biological fluids. The application to a specific biomarker would be the subject of our next investigation to continue this study.

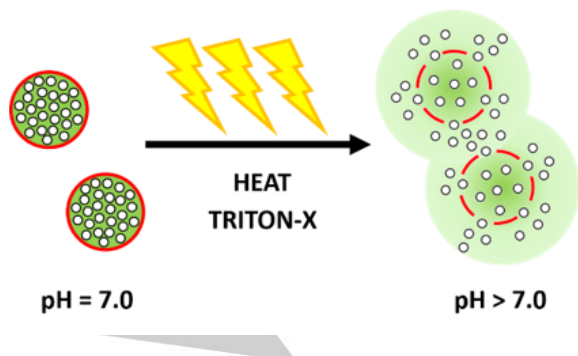
Results and Discussion

Liposomes are vesicles made by phospholipidic bilayers acting as semipermeable membranes. Small lipophilic molecules can cross the bilayer through a diffusion process driven by a concentration gradient (e.g. doxorubicin).^[17] Viceversa hydrophilic ions are not expected to cross the phospholipid bilayer^[18] and this was the working hypothesis of the herein proposed method as it requires that the liposome membrane has to be impermeable to H_3O^+ and OH^- ions (to guarantee the pH gradient between intra- and extraliposomal compartments). The first step of our project dealt with a careful control of the impermeability of the liposomal membrane to the electrolytes used to generate the pH in the inner aqueous cavity.

In the proposed method, the aqueous core of the liposomes was loaded with strong or weak basic substances.

To this purpose, different LipHosomes, either SUVs or GUVs, entrapping NaOH 1 mM (in NaCl 0.15 M) or bicarbonate buffer 0.15 M at pH 10, were tested. LipHosomes were prepared through the thin film hydration method (for SUVs) or the "gentle hydration" method (for GUVs)^[19] in presence of the strong base or the buffer (See Supporting Information). The purification from the not entrapped material was carried out by neutralization in case of NaOH (by adding HCl, in NaCl 0.15 M) and by dialysis or ultrafiltration (carried out at pH 7 equilibrated with 0.15 M NaCl) in case of the bicarbonate buffer.

The suspension of purified LipHosomes, isosmotic with the intraliposomal core, is neutral as long as the nanovesicles are intact. Then, LipHosomes were forced to release their content either by adding a surfactant as TRITON-X or by heating the liposomal suspension as sketched in Scheme 1.



Scheme 1. Rupture of LipHosomes with release of their content and consequent variation of pH value.

Whatever is the chosen, upon destroying the liposomes, the released payload induced a variation in the pH value of the medium, measurable by a conventional pH-meter (endowed with a microelectrode suitable for measurements in 96-wells microplates containing 100 μ L of solution), that was correlated to the number of destroyed LipHosomes.

Table 1 reports the LipHosomes formulations investigated in this work.

Table 1. Formulations of the studied LipHosomes.

Name	Membrane	Content
A (LUV)	DPPC/DSPE-PEG2000 95/5	1 mM NaOH - 0.15 M NaCl
B (LUV)	DPPC/DSPE-PEG2000 95/5	150 mM $NaHCO_3$
C (GUV)	DPPC/DSPE-PEG2000 97/3	150 mM $NaHCO_3$
D (GUV)	DPPC/DSPE-PEG2000Biotin 97/3	150 mM $NaHCO_3$

The pH values of the suspensions containing LipHosomes of formulations A, B, and C did not vary over a period of three hours. Formulation C showed to be also stable upon incubation with human serum taken as model of biological fluid, as reported in the supporting information.

LipHosomes encapsulating NaOH

The pH values were calculated by considering that the concentration of OH^- released by a given number of LipHosomes in 1 L of solution is given by Equation 1:

$$\begin{aligned}
 [OH^-]_{released} &= N^\circ \text{ liposomes} \cdot (\text{mol of NaOH})_{single \text{ liposome}} = \\
 &= N^\circ \text{ liposomes} \cdot [NaOH]_{single \text{ liposome}} \cdot V_{inside \text{ liposome}}
 \end{aligned}
 \tag{1}$$

The number of liposomes can be easily converted in moles dividing by the Avogadro's number (NA) and, as the above relationship refers to 1 L of solution, the LipHosomes moles number corresponds to their molar concentration (Equation 2):

$$(N^\circ \text{ liposomes}/NA)/1 \text{ L} = [\text{Liposomes}]
 \tag{2}$$

The proposed method is based on a pH readout before and after triggering the full release of the inner payload from the LipHosomes. The initial pH should be close around neutrality, but the release of the strong base will increase the concentration of OH^- ions in the suspension, thus increasing pH. The measured proton concentration is correlated to the OH^- concentration via the ionic product of water (K_w):

$$[H_3O^+] = \frac{K_w}{[OH^-]_{equilibrium}}$$

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The charge balance of the suspension (considering that NaCl does not affect the calculations) is the following:

$$[\text{OH}^-]_{\text{equilibrium}} = [\text{Na}^+]_{\text{released}} + [\text{H}_3\text{O}^+]$$

$$\text{with } [\text{Na}^+]_{\text{released}} = [\text{OH}^-]_{\text{released}}$$

Combining the two equations above, it is possible to express $[\text{OH}^-]_{\text{released}}$ as a function of $[\text{OH}^-]_{\text{equilibrium}}$:

$$[\text{OH}^-]_{\text{released}} = \frac{([\text{OH}^-]_{\text{equilibrium}})^2 - K_w}{[\text{OH}^-]_{\text{equilibrium}}} \quad (3)$$

and substituting Equation 3 in Equation 1, the pH of the LipHosomes suspension can be correlated to the concentration of the nanovesicles according with the following equation (Eq. 4):

$$\begin{aligned} [\text{LipHosomes}] &= N^\circ_{\text{LipHosomes}} / NA = \\ &= (\text{mol}(\text{OH}^-)_{\text{released}} / \text{mol}(\text{OH}^-)_{\text{single liposome}}) / NA = \\ &= ([\text{OH}^-]_{\text{released}} / ([\text{OH}^-]_{\text{single liposome}} \times V_{\text{intralipo}})) / NA \end{aligned} \quad (4)$$

The sensitivity of the method is of course dependent on the concentration of entrapped OH^- . However, two limitations have to be taken in account, namely: *i*) the iso-osmotic condition for the inner and the outer compartments should hold, in the case of biological samples this value has to be isotonic *i.e.* 300 mOsm/L; *ii*) the ratio between OH^- and phospholipids has to be lower than the saponification value.^[20]

A route to increase the OH^- payload was to consider the Giant Unilamellar Vesicles (GUVs)^[19] (Figure 1).

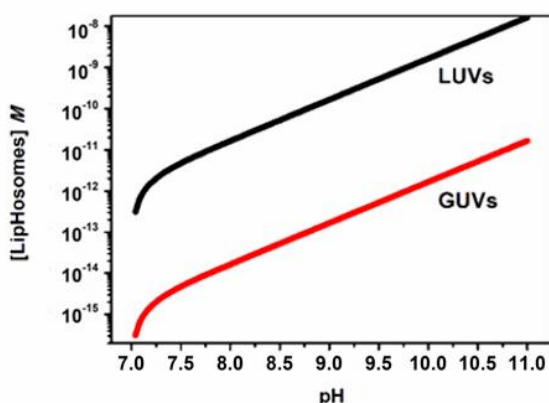


Figure 1. Concentration of OH^- loaded LipHosomes vs. pH generated in the medium upon the release of their payload., calculated on the assumption that *i*) GUVs own a mean diameter of 1 μm and entrap 100 mM of NaOH (red line) and *ii*) LUVs have a mean diameter of 100 nm and entrap 100 mM of NaOH (black line), respectively.

Notably, the sensitivity in terms of LipHosomes concentration using giant LipHosomes reaches the fM range, which appears

very suitable for applications aimed at assaying the detection of the less concentrated biomarkers. In particular, when the comparison is carried out with ELISA-like tests, it is important to note that, in the herein reported approach, the stoichiometry of the interaction between a LipHosome and the analyte is 1:1. Therefore the detection limit of concentration of LipHosomes immediately identifies the threshold sensitivity of the analyte detection. The sensitivity threshold reported for standard colorimetric ELISA tests is in the order of pM, so in principle, this method appears more sensitive. As anticipated in the introduction, the sensitivity threshold of ELISA assays has been pushed further by developing different types of amplification strategies (e.g. magnetic immunoassays^[8], radio immunoassays^[9], liposome-based immunoassays) able to reach the fM concentration threshold. Referring to the above mentioned methods, our approach has the advantage of using much cheaper reagents and technology that can be applied in the field without the involvement of specialized personnel to perform the analysis. On the basis of these preliminary calculations, LipHosomes of Formulation A were tested in a real experiment.

Formulation A (LUVs)

Samples with different concentration of LipHosomes of Formulation A (LUVs) were prepared and independently heated at 55°C for 10 minutes to induce the full release of the liposomal content. Afterwards, the pH of the suspension was measured, and the results are reported in Figure 2.

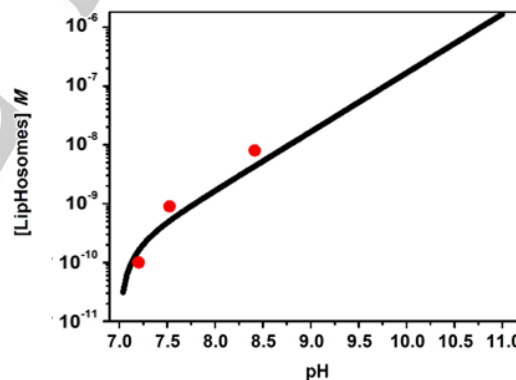


Figure 2. pH dependence on the concentration of LipHosomes after the release of their content. Black line: calculated curve (see above) using the parameters of Formulation A. Red circles: pH readouts for the real experiments with LipHosomes of formulation A.

The pH values measured after the release of the liposomal payload are quite close to the calculated values. However, the measured values were lower than expected for the two more concentrated samples, while in the less concentrated sample the pH was slightly higher.

The discrepancies in pH measurements have to be related to the fact that the pH readout was not carried out in a buffered solution, *i.e.* the pH resulted very unstable over time as a consequence of the slow acidification associated to the progressive CO_2 dissolution in the solution. To avoid such pH instability, we deemed useful encapsulating a basic, but buffered, solution, *e.g.* sodium bicarbonate, in the LipHosomes.

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LipHosomes encapsulating sodium bicarbonate

As the theory anticipates different dilution effect of the released salt upon the LipHosomes concentration, the simulation of the pH dependence on the concentration of LipHosomes encapsulating a sodium bicarbonate buffer appears rather difficult due to the complexity of the multiple equilibria present in solution.

The experiment was carried out as follows: *i)* a 150 mM solution of sodium bicarbonate was prepared, *ii)* the solution was basified with NaOH to pH 10.0, *iii)* the solution was diluted with water several times in the range comprised between 2.5×10^{-2} M and 1.25×10^{-6} M, *iv)* the pH was measured after each dilution.

The salt concentration was expressed as $[\text{HCO}_3^-]_{\text{nominal}}$ which represents the nominal amount of bicarbonate, *i.e.* the pH dependent transformation of HCO_3^- in CO_2 and CO_3^{2-} ion was not considered.

The obtained data (Figure 3) displayed a region (0.01 - 1 mM) in which the pH readout (that ranges from 7.5 to 9.5) is very sensitive to the salt concentration. At concentrations higher than 1 mM and lower than 0.01 mM the pH is almost independent of the salt concentration.

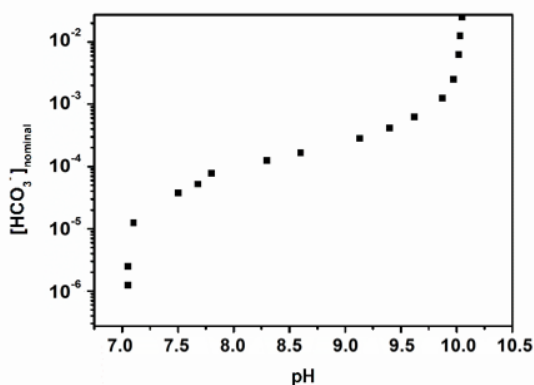


Figure 3. Nominal concentration of NaHCO_3 in solution vs. pH.

Once known the mean diameter of the vesicles and the concentration of their payload, in analogy to what done above in the case of NaOH encapsulation, the number of releasing LipHosomes (in 1 L of suspension) can be correlated to the concentration of the released HCO_3^- as shown by Equation 5:

$$\begin{aligned}
 [\text{HCO}_3^-]_{\text{released}} &= N^\circ \text{ liposomes} \cdot (\text{mol of } \text{HCO}_3^-)_{\text{single liposome}} = \\
 &= N^\circ \text{ liposomes} \cdot [\text{HCO}_3^-]_{\text{single liposome}} \cdot V_{\text{inside liposome}} \\
 [\text{LipHosomes}] &= N^\circ \text{ LipHosomes} / NA = \\
 &= (\text{mol}(\text{HCO}_3^-)_{\text{released}} / \text{mol}(\text{HCO}_3^-)_{\text{single liposome}}) / NA = \\
 &= ([\text{HCO}_3^-]_{\text{released}} / ([\text{HCO}_3^-]_{\text{single liposome}} \times V_{\text{intralipo}})) / NA
 \end{aligned}
 \tag{5}$$

Figure 4 displays this correlation, after substituting $[\text{HCO}_3^-]_{\text{nominal}}$ to $[\text{HCO}_3^-]_{\text{released}}$, for both LUVs and GUVs formulations, which confirms the difference already observed in the formulations loaded with NaOH.

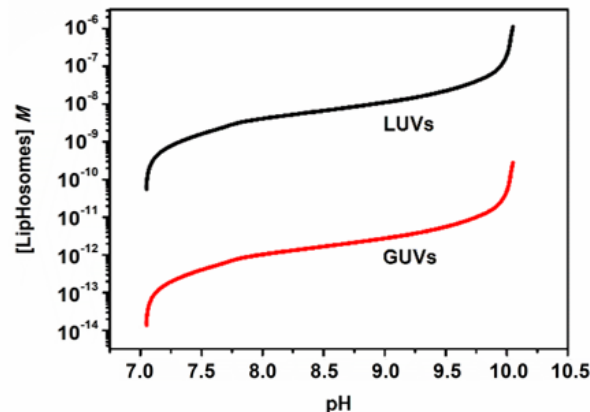


Figure 4. pH dependence on the concentration of bicarbonate loaded LipHosomes, for GUVs (diameter 1 μm , red line) and LUVs (diameter 100 nm, black line) entrapping 150 mM of NaHCO_3 .

Furthermore, the data reported in Figure 4 clearly indicates that the validity range of this method in terms of LipHosomes concentration is spanned over two orders of magnitude (0.1 - 10 nM for LUVs and 0.1-10 pM for GUVs), instead of the 4 orders determined for the LipHosomes loaded with a strong base (Figure 1).

After having experimentally simulated the expected pH values following the release of LipHosomes loaded with bicarbonate at pH 10, real release experiments were planned and executed using formulations B and C.

Formulations B (LUVs) and C (GUVs)

Samples containing different concentration of LipHosomes of formulation B (LUVs) were prepared and independently heated at 55°C for 10 minutes to induce the full release of the liposomal payload. Afterwards, the pH of the suspension was measured, and the results are reported in Figure 5 (batch 1, magenta triangles). The same plot reports the results obtained using a second and a third batch of formulation B of LipHosomes (green triangles and orange triangles, respectively).

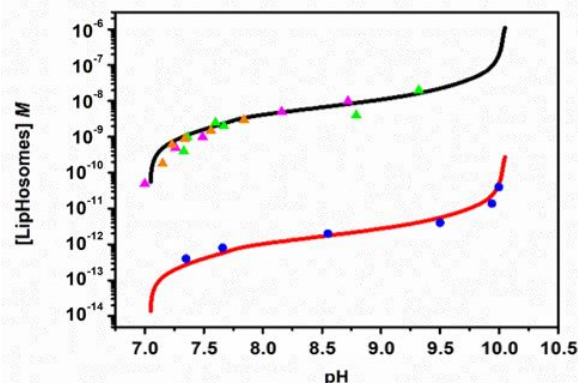


Figure 5. pH dependence on the concentration of LUVs and GUVs LipHosomes loaded with bicarbonate. Magenta triangles: LipHosomes of formulation B, batch 1 (LUVs). Green triangles: LipHosomes of formulation B, batch 2 (LUVs). Orange triangles: LipHosomes of formulation B, batch 3 (LUVs). Black line: calibration data for liposomes with similar size and composition of formulation B. Blue circles: LipHosomes of formulation C (GUVs). Red line: calibration data for liposomes with similar size and composition of formulation C.

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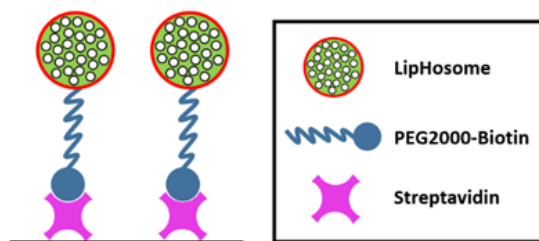
The over imposition of the experimental data with those showed in Figure 4 for LUVs with similar size, shows a good agreement, as well as a good inter-pH reproducibility.

The same procedure (except for the triggering stimulus, in this case operated by the addition of TRITON-X) was applied for the formulation C, GUVs loaded with bicarbonate 150 mM at pH 10.

The excellent agreement between experimental and calculated data (Figure 5) confirmed the good reliability of this approach.

Validating the method in a ligand/anti-ligand test

LipHosomes are expected to find application as reporters for a number of *in vitro* diagnostic tests based on the ligand/anti-ligand binding. Herein we report preliminary observations obtained by using a biotinylated LipHosome designed to recognize streptavidin molecules deposited on the bottom of a plate. Scheme 2 illustrates the ligand/anti-ligand assay taken as test in this work.



Scheme 2: Example of the direct ligand/anti-ligand assay using LipHosomes tested in this work.

In this case, the assay consisted of the direct binding between the anti-ligand, immobilized on a plate, and the properly functionalized LipHosome. The biotin/streptavidin association is at the basis also of commonly applied indirect assays where the ligand/anti-ligand recognition occurs via a third molecular partner that is able to bind both ligand and anti-ligand. Thus, the sensibility threshold from the herein reported experiment is of general applicability.

The analyte anti-ligand to be detected is streptavidin which binds directly to the ligand molecules (biotinylated LipHosomes), to form ligand/anti-ligand binding complexes. In the reported experiment streptavidin is already adsorbed on a 96-well microplate.

Giant LipHosomes containing a biotinylated phospholipid on their membrane and 150 mM bicarbonate in the inner aqueous cavity were prepared (Formulation D). Freshly prepared LipHosomes at a concentration of about $1 \cdot 10^{-11}$ M, were incubated in the microplates for 15 minutes. Then the microplates were washed with NaCl 0.15 M at pH 7.0. A solution of TRITON-X at pH 7.0 was added to the microplates and the pH was measured in continuous for 15 minutes. Full release of the vesicle content was achieved 1 minute after the addition of the surfactant. These data allow us to conclude that the herein proposed method is a fast-responding one. A 0.2 units increase of pH was measured. According to calibration curve reported in Figure 4, this value indicates that about $1 \cdot 10^{-13}$ M of LipHosomes were bound to the plates.

This experiment was repeated five times and results are reported in Figure 6 (Mean \pm SD). As control, the same experiment was carried out using Giant LipHosomes of analogous size and membrane composition (Formulation C) but deprived of biotin moieties on the external surface. In the latter case the pH variation resulted to be about 0.05 units. As further control, microplates were added with washing solution alone (NaCl 0.15 M pH 7.0). The controls were repeated 5 times and results are reported in Figure 6 (Mean \pm SD).

The statistical analysis of the results showed that the difference between functionalized and control LipHosomes is statistically significant (unpaired two-tails T student test, $p = 0.0002$).

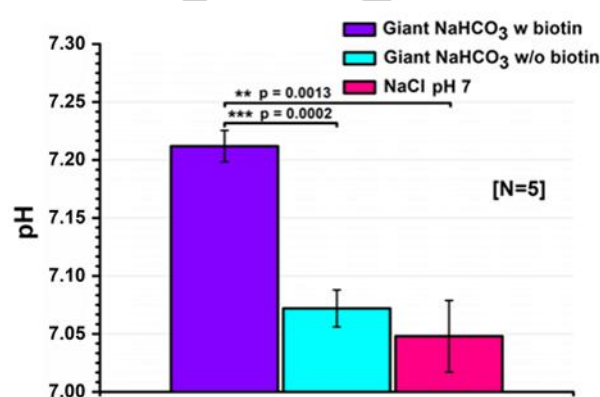


Figure 6: pH variation after direct ligand/anti-ligand assay using giant LipHosomes with biotin (Formulation D, Violet), giant LipHosomes without biotin (Formulation C, Cyan) and washing solution of NaCl 0.15 M pH 7 (Pink).

Conclusions

LipHosomes represent a platform of reporters that may be used to design many low costs dosing tests. Herein a proof of concept of their potential efficacy in ELISA-like tests where the analyte is immobilized on a solid plate is reported. It has been shown that the markedly high sensitivity shown by the use of GUVs may pave the way to a new generation of highly sensitive dosing method based on pH reading. The main advantage of the proposed test over other electrochemical immunoassays, is represented from i) the use of reagents much cheaper rather than enzymes and ii) from the rapidity of the response (1 minute). Moreover, electrochemical methods based on pH readout have the advantage over other dosing tests of making use of an easy to handle and low cost instrumentation, almost present in every analysis laboratory and that doesn't require highly qualified personnel to perform the analysis. For the above reasons we believe that LipHosomes would represent a very interesting platform for the development of new dosing method that can be applied to a wide range of diagnostic biomarkers.

Keywords: dosing methods • encapsulation • immunoassays liposomes • reporters

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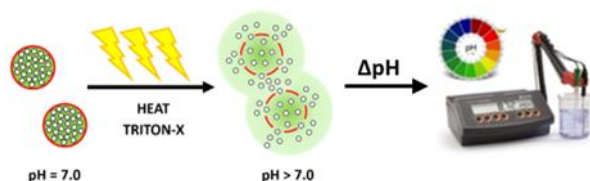
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RESEARCH ARTICLE

Table of Contents



LipHosomes are liposomes designed to induce a change of pH upon releasing their content. The release of the liposomal content exhibits a quantifiable variation of the pH of the medium. The biotin-streptavidin binding scheme was exploited as the model for analytical assay application, showing an excellent sensitivity being able to reveal a concentration of bound LipHosomes in the sub-pM range.