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Technical Note

An Electrochemical Strip to Evaluate and to Discriminate Drug Encapsulation in Lipid Nanovectors

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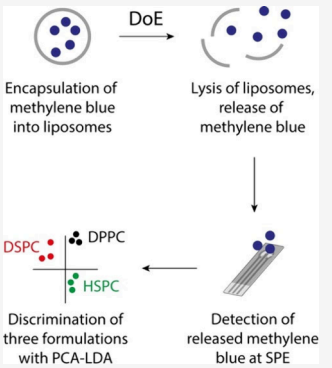
ABSTRACT: Lipid nanovectors (LNVs) represent potent and versatile tools in the field of drug delivery for a wide range of medical applications including cancer therapy and vaccines. With this Technical Note, we introduce a novel "portable", easy-to-use, and low-cost strategy for double use: (1) it allows one to both quantify the amount of cargo in LNV formulation and (2) classify the nature of formulation with the aim of chemometrics. In particular, an electrochemical strip, based on a screen-printed electrode, was exploited to detect methylene blue (MB) as the model cargo encapsulated in various liposomes (used as model LNV). The experimental setup, including release of the MB content and its electrochemical quantification were optimized through a multivariate design of experiment (DoE), obtaining a satisfactory 88−95% accuracy in comparison to standard methods. In addition, the use of principal component analysis-linear discriminant analysis (PCA-LDA) highlighted the satisfactory differentiation of liposomes. The combination of portable electroanalysis and multivariate analysis is a potent tool for enhancing quality control in the field of pharmaceutical technologies, and also in the field of diagnostics, this approach might be useful for application toward naturally occurring lipid nanoparticles, i.e., exosomes.



INTRODUCTION

Lipid nanovectors (LNVs), including liposomes and lipid nanoparticles, have emerged as revolutionary players in the field of drug delivery,[1] presenting a versatile and efficient platform that addresses critical challenges associated with traditional therapeutic approaches.[2,3] Given the versatility in encapsulating a broad spectrum of both hydrophobic and hydrophilic pharmaceutical compounds, LNV formulations are under the spotlight for a wide range of medical applications, including cancer therapy,[4] gene therapy,[5] and vaccines[6] due to higher biocompatibility and pharmacokinetics ability. The encapsulation of therapeutic agents also facilitates targeted and controlled delivery, contributing to the optimization of therapeutic outcomes.[7−9] Drugs loaded into liposomes become bioavailable only when they are released. Therefore, to achieve optimal therapeutic activity, it is necessary to effectively encapsulate, monitor, and modify the rate of drug release from liposomes. On the other hand, the high loading into the LNV is mandatory to administer a limited dose of the formulation.

In the evaluation phase of a pharmaceutical formulation, a systematic and methodical approach is essential to evaluate parameters such as encapsulation efficacy, stability,[10] and safety, due to decision-making processes regarding further optimization, scale-up, and potential clinical applications.[11−13] Quantifying the loaded cargo is a multifaceted process that involves various analytical methods,[10,14−16] including High-Performance Liquid Chromatography (HPLC), UV−vis spectroscopy, and mass spectrometry. All of these methods allow analysts to assess the encapsulation efficiency, ensuring that liposomes carry the intended amount of therapeutic cargo.[17−20] However, despite all of the available analytical approaches, the evaluation of the liposome encapsulation efficacy is routinely performed at the end of the whole process. In order to avoid both laborious procedures and time/reagent loss (in the case of synthetic issues during their production), the development of facile and low-cost analytical procedures, as represented by portable analytical methods, i.e., sensors and biosensors, might represent a valuable alternative:[21] these might be consistent with a fast monitoring of liposomes' quality during synthesis, also improving scalability and the quality of encapsulation: electrochemical sensors and biosensors, via screen-printed electrode (SPEs), might represent valid candidates as demonstrated by their application in various fields of biomedicine.[22−24] The most valuable features of SPEs are represented by their cost-effectiveness, disposability, portability, and easy customization.[25] To date, the adoption of SPEs associated with liposomes has been majorly applied for developing sensing architectures as reported in the literature. For instance, a liposome composed mainly of dipalmitoyl-

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phosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and cholesterol was filled with ferrocene carboxylic acid and used as an electrochemical label for the development of an immunoassay for the detection of carcinoembryonic antigen in saliva: in this case, after the antibody−antigen recognition was obtained, the liposome was dissolved in a methanol-triton X-100 mixture and then analyzed with voltammetry experiments.[26] A similar liposomal architecture, loaded with methylene blue, was also exploited as an electrochemical label in the development of an electrochemical immunosensor for progesterone detection: in this case, the released electrochemical mediator was measured after liposomes were vacuum-dried.[27] Another work exploited the role of a methylene blue-loaded liposome as an electrochemical label to detect enzyme phospholipase A2. The release of the electrochemical mediator, and its following detection, was activated by the presence of the target enzyme and its ability to hydrolyze the phospholipid of the liposomes.[28] The same approach based on the use of liposomes as the electrochemical labels has also been reported for the development of other sensing architectures.[29] However, all these works exploit the use of liposomes as electrochemical labels to transduce recognition events, e.g., antibody−antigen, enzyme−substrate, nucleic acid hybridization, while the aim of this Technical Note is to highlight the development of an electrochemical approach to quantify and characterize the efficacy of cargo encapsulation. In order to provide an efficient tool for quality control, three lipid nanovectors containing cholesterol and distearoylphosphatidylcholine (DSPC), DPPC, and hydrogenated soybean phosphatidylcholine (HSPC) have been loaded with methylene blue and subsequently analytically characterized, including the use of a multivariate design of experiment (DoE) to optimize methylene blue (MB) detection and principal component analysis-linear discriminant analysis (PCA-LDA) to characterize and classify each liposome formulation. This work presents the proof-of-concept application of an electrochemical device in the LNV field, representing a starting point for supporting application in the pharmaceutical quality control process.

EXPERIMENTAL SECTION

Reagents. Cholesterol (Chol), methylene blue (MB), and Triton X-100 was provided by Sigma-Aldrich (St. Louis, MO, USA). The lipids distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), and hydrogenated soybean phosphatidylcholine (HSPC) were produced by Lipoid GmbH (Ludwigshafen, Germany). Methanol and other solvents were obtained from Exacta Optech (Italy).

Liposome Preparation and Quantification of Encapsulated MB. MB-loaded liposomes were prepared by hydration of a lipid film followed by extrusion.[30,31] In the first step, different lipids were dissolved in a chloroform/methanol solution (2:1 v/v) and mixed in a 50 mL glass flask. The thin film was obtained by a rotary evaporator (Laborota 4010 digital, Heidolph, Schwabach, Germany) and then hydrated with a methylene blue aqueous solution (0.5 mM) for 1 h. Depending on the lipid composition, different hydration temperatures were used. The liposome suspension was then extruded by a thermobarrel extruder system (Northern Lipids Inc., Vancouver, BC, Canada) repeatedly passing the suspension under nitrogen through polycarbonate membranes with decreasing porosity (0.4 and 0.2 μm; NucleoporeTrack, Whatman, Brentford, UK). Methylene blue-loaded liposomes were dialyzed in water to remove the nonencapsulated methylene blue. The liposome formulation was stored at 4 °C. All formulations developed are reported in Table S1, Supporting Information. Blank liposomes were also prepared and used as a control. All the physical-chemical characterizations of the liposome formulations are reported in the Supporting Information file (Figures S1 and S2). To measure the amount of MB encapsulated in the formulation, liposomes were dissolved in methanol (1:100 v/v), and samples were centrifuged for 20 min at 13 000 rpm and 25 °C using a laboratory centrifuge D1524R (DLAB Scientific Co., Ltd., China). Supernatants were then spectrophotometrically analyzed at 653 nm.

Electrochemical Measurements on Electrochemical Strips. The electrochemical measurements were carried out with a commercial carbon SPE (DropSens 110, Metrohm, Italy) and μStat portable potentiostat (Metrohm). Cyclic voltammetry was first performed to understand the potential of MB released from different formulations, and for this purpose, the large range was tested (−0.9 to 0.9 V) with an "Estep" of 0.02 V and scan rate of 0.05 V/s. All measurements were performed in the presence of potassium chloride (KCl) as the supporting electrolyte at a concentration of 0.1 M. A DSPC was used as a model formulation, and the faradaic peak was recorded at range of ca. −0.3 V. The measurements were performed in linear sweep voltammetry (LSV) mode from −0.5 to 0 V. The parameters in terms of lysis protocol and time of measurement acquisition are reported in another subparagraph. DPPC, DSPC, and DPPC were tested in LSV mode. For each formulation, the calibration curves were acquired by using the same formulations of blank (without MB enclosed) spiked with MB at increasing concentration ranges from 2 to 12 μg/mL in the presence of Triton X-100 1% and KCl 0.1 M following the optimized condition of DoE results. The same method was performed for MB quantification encapsulated in different formulations. The data from the electrochemical experiments were acquired with DropView 8400 Software and analyzed with CAT software.[32]

MB Release and Electrochemical Optimization with DoE. To evaluate the correct experimental procedure involving both the lysis of the liposomes and the measurement of the released MB, we used the DoE. In particular, we investigated the main variables such as the temperature at which the lipid to carry out the melting point occurs lipids, the concentration of the surfactant used to facilitate the opening of the bilayer, the incubation time, and the deposition time of the drop on the electrode. A D-Optimal design model was used, described in the following equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3b_4x_4 + b_{12}x_{12} + b_{13}x_{13} + b_{14}x_{14} + b_{23}x_{23} + b_{24}x_{24} + b_{34}x_{34}$$

In detail, we investigated three different temperatures such as 50, 60, and 70 °C for three different incubation times 15 min, 30 min, and 1 h in the absence and presence of triton X-100 at two different concentrations of 1% and 2%. To establish an electrochemical measurement that considers the surface interaction of the working electrode, we tested the interaction time of the liposome drop in a time range from 5 to 20 min.

MB Quantification and PCA-LDA for Classification of Different Types of Formulation. To measure the content of MB loaded within each formulation, the samples were incubated with 1% of Triton X-100 for 1 h at 60 °C for

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complete lysis. A 50 μL drop was spotted on the carbon surface of the SPE, stabilizing the interaction of the released MB with the working electrode for 20 min, and the electrochemical measurements were carried out with SPEs in linear sweep voltammetry (LSV). To discriminate the lipid nanoparticles, the voltammograms of each formulation were tested by carrying out cross-validation tests with the application of PCA-LDA via the software CAT. Figure 1 shows the whole workflow which includes: MB encapsulation, DoE optimization of liposomes' lysis and MB electrochemical detection at the printed strip, and PCA-LDA classification of the liposomes' formulation.

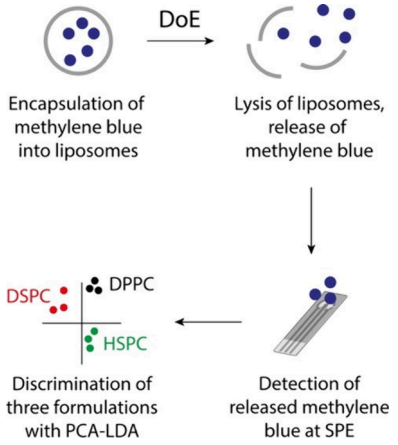

Figure 1. Workflow of the whole experimental setup.

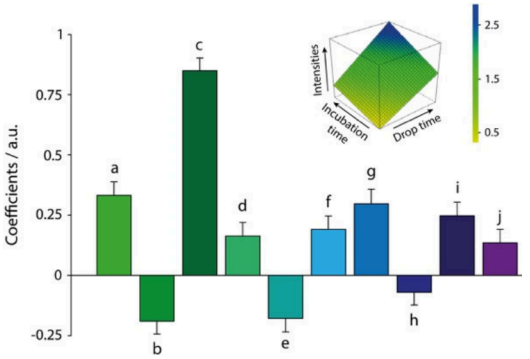

Figure 2. DoE results obtained from the analysis of electrochemical experiments. Histogram represents the weight intensity detected in the evaluation of the experimental variables (a–j) which are (a) incubation time, (b) triton X-100 concentration, (c) drop time on the strip, and (d) temperatures. The first-order interaction of parameters is reported as (e) incubation time-triton X-100 concentration, (f) incubation time-drop time, (g) incubation time-temperature, (h) triton X-100-drop time, (i) triton X-100-temperature, and (j) drop time-temperature. In the inset, a 3D graph shows the response surface between the variables that show the highest contribution in the sensor development, incubation time, and drop time. The z-axis represents the intensity of the sensor response in terms of μA, predicted by the model at each point in the experimental domain.

RESULTS AND DISCUSSION

Optimization of the Experimental Setup. In order to obtain the optimal experimental conditions to both open the liposomes and detect the contained MB at the screen-printed electrode, the DSPC-based liposomes were used as the model formulations. In particular, due to the diverse parameters to be evaluated, a design of experiment (DoE) has been taken into account. In fact, the adoption of a multivariate strategy has allowed us to consider all the contributions and the interactions among parameters of different natures and also to obtain an optimization with the use of a lower number of experiments. In particular, the following parameters have been considered: incubation time, triton X-100, temperature, and drop time. Briefly, incubation time was consistent with the time of contact between liposomes and triton X-100; triton X-100 was the concentration of the surfactant. Temperature was the heating temperature, and drop time was consistent with the time to leave the drop onto the electrode prior to be electrochemically analyzed. In addition, thanks to the DoE, D-Optimal and also the first-order interaction among the various parameters were taken into consideration, e.g., triton X-100-temperature, drop time-incubation time, etc. As above-mentioned, all the experiments were carried out through the use of LSV to detect the amount of released MB: as shown in Figure 2, the contribution of each parameter was evaluated on the electrochemical response.

The DoE allowed researchers to study the effect of all the experimental parameters with the performance of 57 experiments instead of 108 as estimated by performing a one variable at a time (OVAT) approach.[33,34] At the levels, three temperatures were evaluated 50, 60, and 70 °C, three incubation times of 15, 30, and 60 min, and three levels per triton X-100 0, 1%, and 2%, and to improve the electrochemical readout, a drop time from 5 to 20 min was considered. Three replicates were performed for each condition with a total of 19 combination groups tested. As a result of these experiments, Figure 2 reports the "weight" of the variable and a combination of these in the SPE measurements. The main parameters that showed the greatest influence on voltammetric measurements were the drop time and the incubation time of the formulation to release all the o contained. With regard to the drop time, meaning the time of contact between the lysed liposomes with the electrode, it appeared to have the highest influence. This behavior could be ascribable to the adsorption of MB on the carbon-based working electrode, as also reported in the literature.[24] The incubation time was also characterized by a higher intensity with respect to other parameters: how the prolonged interaction with the surfactant might facilitate the release of MB is reasonable. In addition, the coefficient of variation (CV) was calculated as the percentage value of the ratio between the standard deviation and the mean peak oxidation current obtained from three different replicates performed for each tested condition. Figure S3 in the Supporting Information shows the CV% results for the different LNVs, always lower than 3% (five replicates).

Release of Liposomal Cargo and Electrochemical Quantification. Following the optimization study that was carried out, the electrochemical quantification of the released liposomal cargo, i.e., methylene blue, was performed on three formulations, namely, DSPC, DPPC, and HSPC, using the following experimental parameters: triton X-100 1%, temperature 60 °C, incubation time 1 h, and drop time of 20 min. We optimized these parameters on the basis of the results obtained from the DoE experiment, with some modifications. All the electrochemical measurements were performed on a carbon-based screen-printed electrode in the presence of KCl 0.1 M,

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and the calibration and formulation voltammogram are reported in Figure 3.

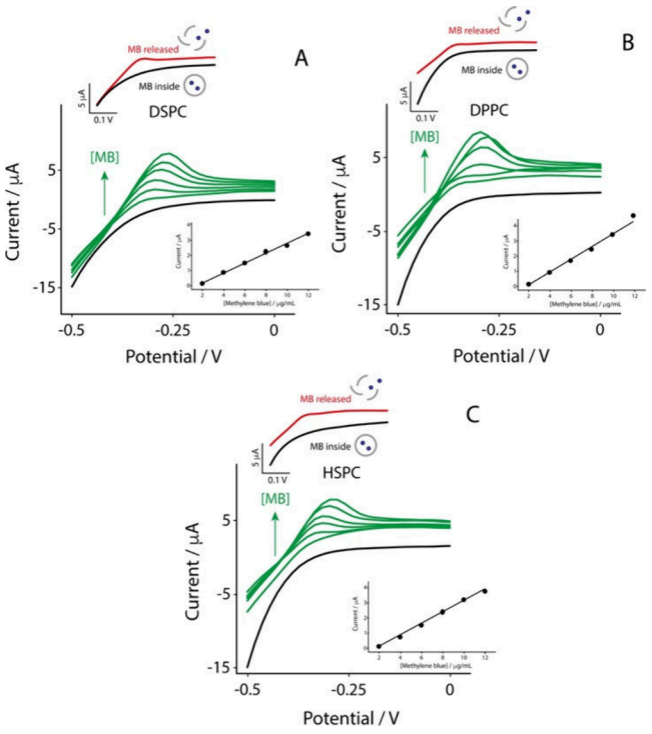

Figure 3. Voltammetric curves obtained with LSV and the corresponding calibration curve from 2 to 12 μg/mL of methylene blue (green curves). LSV curve before (black curve) and after the lysis procedure (red curve) for (A) DSPC, (B) DPPC, and, (C) HSPC liposomal formulation, respectively.

For all of the formulations that have been considered, MB-spiked blank liposomes (in the range between 2 and 12 μg/mL) have been electrochemically analyzed. This step was necessary to evaluate the sensitivity toward MB in the various formulations: in particular, very satisfactory correlations were obtained as described by the following calibration curves, namely, $y = 0.32x + 0.47$ ($R^2 = 0.99$), $y = 0.42x + 0.62$ ($R^2 = 0.99$), and $y = 0.38x + 0.63$ ($R^2 = 0.99$), respectively, for DSPC, DPPC, and HSPC. The maximum oxidation peak was recorded at −0.3 V for all of formulations tested. As shown in the upper inset of Figure 3A−C, it is obvious how the signal due to the oxidation of MB appeared when the MB-containing liposomes were lysed following the optimized procedures. In particular, the content of MB was quantified, and the accuracy was obtained by comparing the electrochemical results with the spectrophotometric one at 653 nm. The quantification measurements showed loadings of 5.7, 5.2, and 5.2 in μg/mL, respectively, for DSPC, DPPC, and HSPC compared to the standard method, which revealed loadings of 5.4, 4.7, and 4.6, respectively. What should be noted is that the nominal loading was supposed to be 6 μg/mL, and the reported results highlight the importance of developing quick analytical methods to evaluate the "real" concentration of cargo that is delivered for specific treatments. Our method demonstrated an average accuracy of 95%, 90%, and 88%, respectively, for DSPC, DPPC, and HSPC.

Multivariate-Driven Discrimination of Formulations. Although the quantification of the cargo contained in the liposome formulations demonstrated the feasibility of the proposed method, a multivariate analysis technique was applied to identify the formulations by analyzing the voltammogram shapes. In particular, a multivariate statistical technique, namely, principal component analysis-linear discriminant analysis (PCA-LDA), was used to analyze three different data sets of the voltammogram for a total of 60 measurements, with the aim of constructing a discrimination model by using sets of measurements from the three data sets, i.e., DSPC, DPPC, and HSPC, as shown in Figure 4.

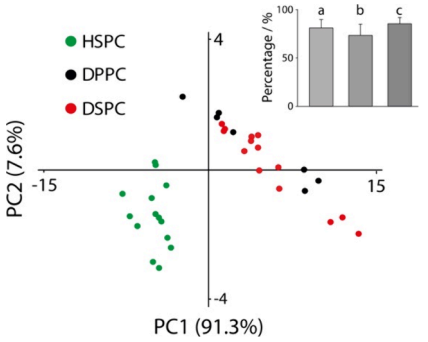

Figure 4. Score plot of the three formulations analyzed and separated with the use of PCA-LDA, including HSPC (green dots), DPPC (black dots), and DSPC (red dots). The inset shows histograms relative to the evaluation of (a) accuracy, (b) specificity, and (c) selectivity.

The PCA-LDA was applied to 60 recorded voltammograms, and the prediction capability was assessed with a cross-validation method. As reported in the literature, the PCA-LDA model is characterized by a high accuracy and specificity in the classification of signals.[35] The score plot reported in Figure 4 is built in the domain of the principal components PC1 and PC2, which carried ca. 98% of all the voltammogram variation found in the whole data set. In particular, the clusters shown in the score plot highlighted the satisfactory differentiations of HSPC and the other two formulations, highlighting two principal patterns based on the shape of the voltammograms with no overlapping score values. In particular, HSPC and the couple DPPC/DSPC are well separated along the PC1, and in fact, HSPC is mainly characterized by negative PC1 score values, while DPPC and DSPC have mainly positive PC1 score values. In particular, the histograms reported in the inset of Figure 4, representing the percentages associated with accuracy, specificity, and selectivity in discriminating voltammograms of the three different compositions, demonstrated a satisfactory total result of ca. 90% prediction accuracy across the three tested formulations.

CONCLUSION

In this Technical Note, for the first time, we present a portable electrochemical platform to be applied for improving quality control during encapsulation of lipid nanoparticles for drug delivery application. A double use of this tool is recognized: the possibility to quantify the cargo and the effectiveness in defining the quality of vectors with the adoption of chemometrics. Although the exploitation of lipid nanoparticles for delivering drugs and vaccines has increased in recent years, the evaluation of the encapsulation efficacy is still carried out with the use of analytical techniques characterized with multiple tasks and specialized equipment, i.e., chromatography, spectrophotometry, etc. The design, characterization, and

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application of a low-cost and easy-to-use protocol, based on the adoption of screen-printed electrodes, have highlighted the possibility to quantify the efficacy of cargo encapsulation within liposomes. As a model redox substance, methylene blue was encapsulated into liposomes, and the proposed electrochemical method yielded an accuracy higher than 90% in comparison with the spectrophotometric determination used as the gold standard. Furthermore, the coefficient of variation obtained is less than 3% in the quantification of the encapsulated analyte in the three lipid formulations. Moreover, the use of multivariate analysis, i.e., principal component analysis coupled to linear discriminant analysis, demonstrated the possibility in differentiating three formulations loaded with methylene blue with varying lipid composition. The classification was possible as the voltammetric curves for each formulation were characterized by different shapes, thus confirming the electrochemical detection as a powerful tool for their discrimination and as a proof of concept for automated analysis. The development of this method represents a valuable tool for quality control in pharmaceutical technology applications. It is important to note that each lipid formulation varies in its liposomal composition and encapsulated content. Therefore, it is crucial to test the method for each variable present and also employ reference techniques for validation.

This study paves the way for a versatile tool in the field of drug delivery for many reasons: (i) it might be adopted while cargo are encapsulated into liposomes to evaluate the efficiency, by quantifying the real cargo concentration without the use of sophisticated techniques, and in real-time, (ii) in combination with multivariate analysis, the electrochemical detection at the screen-printed electrodes is capable of providing a classification to differentiate liposomes on the basis of the voltammetric curves' profile, and (iii) the same architecture might be used in the future for diagnostics, e.g., the quantification of biomarkers within circulating extracellular vesicles associated with cancer or other diseases, the differentiation among healthy and unhealthy patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c01997.

Table S1, Figure S1, and Figure S2 focused on the characterization of lipid nanovectors; Figure S3, information about coefficient variation calculation (PDF)

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Notes

The authors declare no competing financial interest.

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