31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Interactions of Nanoparticles with Lipid Vesicles: A Population Based Computer Aided Image Analysis Approach

Jernej Zupanc, *Student Member IEEE*, Janez Valant,
Andrej Dobnikar, Veronika Kralj-Iglič, Aleš Iglič, Damjana Drobne

***Abstract*—Novel properties of nanoparticles have numerous potential technological applications but at the same time they underlie new kinds of biological effects. Uniqueness of nanoparticles and nanomaterials requires a new experimental methodology. Much evidence suggests that nanoparticles affect cell membrane stability and subsequently exert toxic effects. For this kind of research, lipid vesicles are of high value due to controllability and repeatability of experimental conditions. The aim of work presented here was to develop a computer aided analysis of lipid vesicles shape transformations. We studied a population of palmitoyloleoylphosphatidylcholine (*POPC*) lipid vesicles after exposure to nanoparticles (C_{60}) or a reference chemical ($ZnCl_2$). With the use of computer image analysis methods, we detected differences in size distributions of vesicles in different exposure groups. Though, at the present state, we are not able to precisely identify effects of nanoparticles on shape transformations of vesicles, those incubated with nanoparticles were in average larger than those in other groups. This population based approach holds many promises for future investigation of nanoparticles—lipid vesicles, or even nanoparticles—biological membranes interactions. However, in order to get reliable results, numerous images have to be analyzed which requires improved and highly automated image segmentation and analyses methods.**

I. Introduction

FROM a chemistry and material science perspective, the development of new products using nanomaterials is exciting because, for a given particle-type, as one moves down the nanoscale (i.e., as the particle size is decreased within the nanoscale range), fundamental physical and chemical properties appear to change. Novel properties which are distinguishing nanoparticles from the bulk material typically become apparent at critical particle lengths below 100 nm. Particles of this size have numerous potential technological applications but may be hazardous for biological systems. Wide use of nanotechnology means that more and more man-made nanoparticles could in their life-time enter our atmosphere, soil or water environments.

Recent studies report that the toxicity of some nanoparticles may be in a large part related to their surface reactivity. Much evidence suggests that nanoparticles can effectively disrupt cell membranes by formation of nano scale holes, membrane thinning, and peroxidation. Many recent papers report results of *in vivo* and *in vitro* effects of nanoparticles on cell membrane stability [1][2][3][4][5]. It appears that lipid vesicles, which are simplified biological membranes, offer controllable conditions to study effects of xenobiotics and can easily be observed directly under light microscope. Conformational behavior of vesicles is also very frequently studied [6][7][8][9], but most of the studies focus only on observing a single vesicle and its transformations during interaction with nanoparticles. However, as fluctuation of membrane shapes is a very dynamic process, we believe that even more information about the interactions could be obtained by observing populations of vesicles.

In the study presented here, an experimental set up is elaborated, which is based on analyzing populations of vesicles exposed to nanoparticles or a reference chemical. Instead of focusing on a single vesicle, we acquire multiple images of subsets of vesicle population after different time exposures. This approach allows us to capture the dynamics of shape transformations on a completely new scale. We investigated if shape transformations (size, eccentricity) and differences in quantity of vesicles can be accessed through this population based approach.

The aim of work presented here was to develop a methodology, together with a computer aided analysis of lipid vesicles shape transformations.

II. Materials and Methods

A. Experiment

Lipid vesicles were suspended in 45µl of 5% glucose. This suspension was divided into three parts, applied on three object glasses and covered with a cover glass. Subsequently, to each of the three glasses, 5µl of either glucose, nanoparticles (C_{60}) in a glucose solution or reference chemical ($ZnCl_2$) in a glucose solution was added on a well defined side of the sample. For the convenience of this paper, we will relate to these samples as control, nanoparticles and reference group. Immediately after adding any of these suspensions or solutions, the vesicles were inspected and multiple images were acquired at three different places on the object glass (Fig. 1). These places were selected to allow us to capture the dynamics of vesicle shape transformations in a gradient of concentrations.

Manuscript received April 23, 2009.
Jernej Zupanc and Andrej Dobnikar are with the Faculty of Computer and Information Science, Veronika Kralj-Iglič is with the Faculty of Medicine, Aleš Iglič is with the Faculty of Electrical Engineering, Janez Valant and Damjana Drobne are with the Biotechnical Faculty Ljubljana, all with University of Ljubljana, Slovenia. (Corresponding author is Jernej Zupanc, phone: +386 1 4768875, e-mail: jernej.zupanc@fri.uni-lj.si).

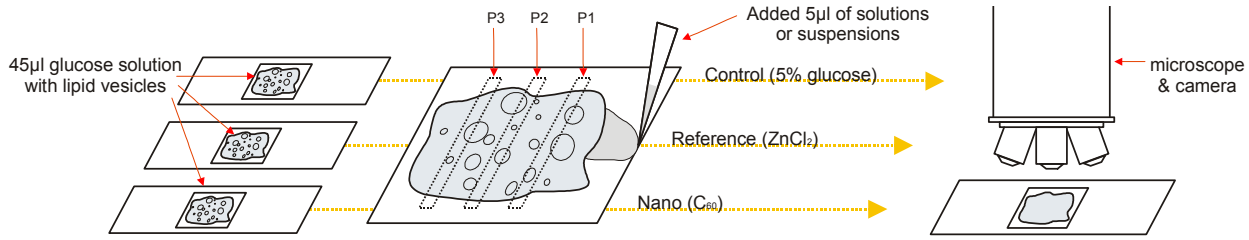

Fig. 1. Sequence of steps in our experiment is presented from left to right. First, equal volumes of glucose solution with lipid vesicles are added to three object glasses. Then, either nanopartiles, a reference chemical or a glucose solution only are added to either of the samples. Three places: P1, P2 and P3 are defined as observing places. Multiple images of these places (in all three samples) are acquired after different time exposures by a camera, attached to the microscope (figure not to the scale).

An inverted microscope (Nikon Eclipse TE2000-S) was used for acquiring the images with 400x magnification. This allowed us to capture vesicles with radii larger than 4µm. The field of view (visible area) of each image acquired at this magnification is 190x143µm. With 10 images in a vertical session we were able to capture approximately 10% of each of the selected places (width and height of each of the three samples on the object glass were 15mm).

First image set of either of three samples was acquired immediately after the solutions or suspensions were added. The second image capturing was 90 minutes later.

B. Image Analysis

All images were labeled so their time, sample group, and place on the object glass could be uniquely identified at any later point. After contrast increasing and noise cleaning filters were applied to the images, the expert used an image editing software to segment all objects of interest (Fig. 2). This is a very important step of our research, as it is the main link between qualitative and quantitative data. Furthermore, much information can be added (or lost) at this point and this is why we based this experiment on images labeled by an expert. Attempts that were already made towards automating the image segmentation required for this research are presented in the next chapter: Automatic Image Segmentation.

A computer aided image analysis was performed to analyze objects in segmented images. At this point of our research, we were mostly focused on size (presented with vesicle radii, since most of them were circular) and quantity of lipid vesicles in the images. However, other properties and other interesting objects could also be segmented in our future work.

When objects were segmented, we were able to link properties of every single lipid vesicle to the sample (control, reference or nanoparticles), incubation time (0 min or 90 min), place on the object glass (P1, P2 or P3), and number of the image, where the object was identified.

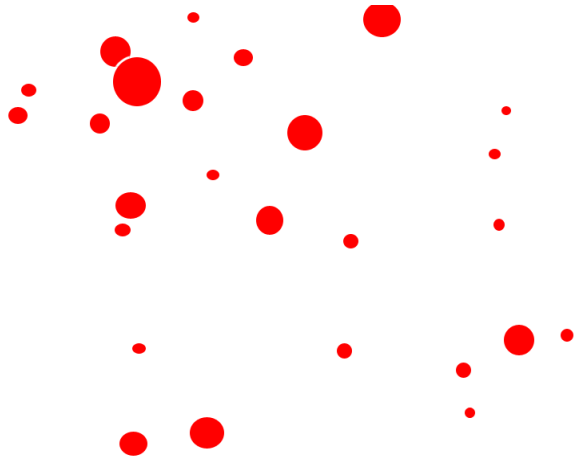

Fig. 2. An example of a labeled image. Objects (mostly circular) represent segmented lipid vesicles. Their properties are evaluated in our computer aided image analysis.

C. Automatic Image Segmentation

Large amounts of highly informative data are necessary for the connections between different features in our experiment to become representative. In this respect, the collected images of vesicle population alone are not informative enough. Currently, the experts add the required information by segmenting images and identifying lipid vesicles. However, this task is very time consuming and it is in our greatest interest to automate this process by incorporating image segmentation and machine learning methods.

As this is an early stage of our research, automatic image segmentation is not yet satisfactory and cannot replace the manual segmentation by experts. Nevertheless, some steps in this direction were already made and the results we achieved are promising for our future work towards fully automated image segmentation and feature analysis approach.

Any data destined for computer aided automatic treatment, has to be gathered with minimal noise. Usually, a smart choice of the data acquiring method contributes more to the accuracy of the final result than post processing and data filtering.

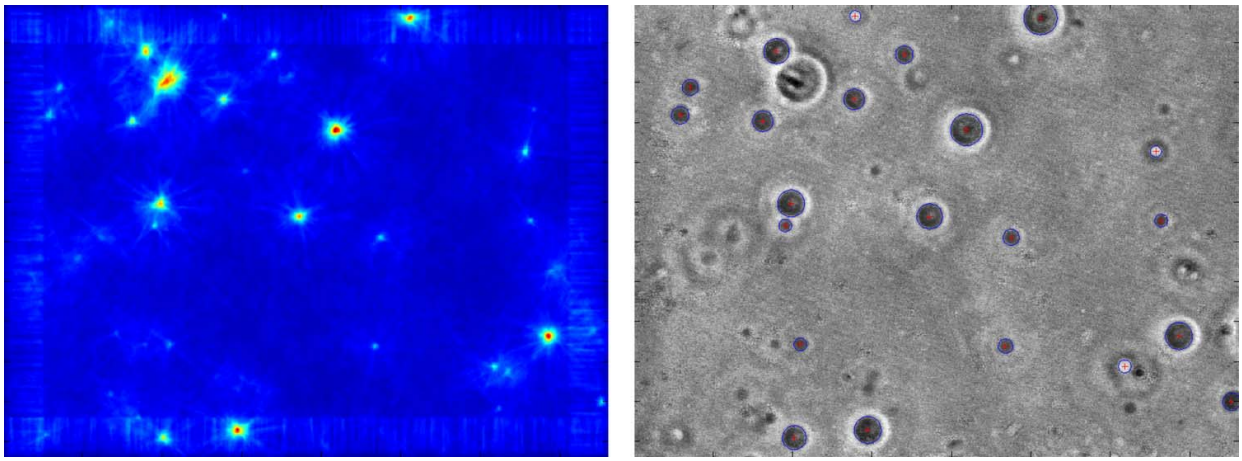

Fig. 3. On the left image, an accumulation of gradient field from Adaptive Hough Transform is shown, where the isolated brighter areas represent regions of interest. On the right image, centers of detected objects (in this case circles) are marked with a plus sign and radii are marked with contours. This is an example of an automated circle detection in our microscopy acquired images of populations of lipid vesicles.

The goal of our image segmentation task was to detect lipid vesicles in images acquired by microscopy. From our previous experiments with individual lipid vesicles and their interactions with various particles, we already had some knowledge about possible shapes of vesicles, yet even more information was gathered during our manual segmentation of images. We learned that shape of lipid vesicles varied from circular (majority) to elliptical. The simplest and the most popular way to separate objects from the background is by thresholding the image intensity. However, this approach did not work for objects in our images, since the lipid vesicles can be of different intensity levels and even their edges are not always clearly visible. Instead, we decided to use the knowledge about the shape of lipid vesicles, and an algorithm for circle detection – a variation of the Adaptive Hough Transform, proposed by Illingworth [10]. This algorithm was previously successfully used by Tao et al. to locate positions of individual micro spheres in images obtained by optical section microscopy [11]. Algorithm takes advantage of the image gradient field, where object boundaries are more determinable compared to intensity field. The image gradient vectors are summed up in an accumulation matrix, where the increased gradients at the edges of circular objects contribute to the accumulation in their centers. A transform is used to convert these gradients into an accumulation matrix (Fig. 3.). In the latter, intensity of each pixel corresponds to the probability of that pixel being the center of a circle. Although such center detection works well for circular lipid vesicles, those with more elliptical eccentricity can still be missed. Our current adaptation of the Hough transform detects approximately 90% of all circles. Since the non-circular lipid vesicles are also a large part of our interest, detecting all elliptical objects (not only circles) is an important goal for our future work.

III. RESULTS

Our results so far showed that nanoparticles have a potential to interact with membranes. This is manifested as size alterations of lipid vesicles. In the experiment presented here, there were notable differences between size distributions of lipid vesicles exposed to nanoparticles compared to other two groups. Although we are currently not able to precisely identify the magnitude of effect C_{60} has on lipid vesicles, we found that when lipid vesicles were exposed to nanoparticles they were in average significantly larger than those that were not (Fig. 4). The two-sample Kolmogorov—Smirnov test was employed to test this hypothesis, which was showed to be correct with confidence level alpha = 0.05 in both cases. All together, 2803 vesicles were detected in images, 1407 in control, 427 in reference, and 969 in nano group.

The current state of our automatic image segmentation is not satisfactory, as on average only 90% of all circular objects are detected. Future work in this area will be focused in adapting methods for circular and elliptical object detection to the lipid vesicles identification task.

IV. DISCUSSION

In the study presented here we show successful acquiring and analysis of image sets of various lipid vesicles populations. Our experiment consisted of exposing lipid vesicles to a reference chemical ($ZnCl_2$) or nanoparticles (C_{60}), acquiring images of the populations and comparing properties of the two exposed lipid vesicles populations.

Our primary interest was in the sizes and quantities of lipid vesicles in each of the compared groups and we were also able to distinguish them in different places and at different incubation periods. At the present state, it is not possible to precisely describe the type and extent of effects of nanoparticles. However, some differences in shapes of nanoparticle—incubated vesicles and controls were also observed. Identifying the shape transformations of vesicles remains our future task, because this could provide data on

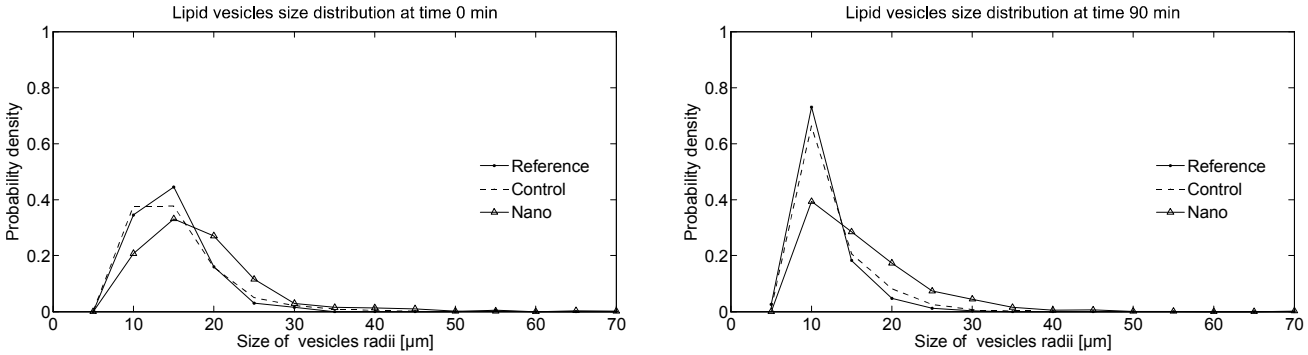

Fig. 4. Both diagrams present a probability density distribution of the radii of lipid vesicles in all three observed groups (control, nanoparticles and reference populations). In the left diagram, we can observe the distributions immediately after the exposure and in the right, the distributions after 90 minutes of incubation. Number of identified vesicles in each group was between 500 and 1500.

the potential of nanoparticles to interact with biological membranes.

Today's imaging technologies generate a wealth of images that require quantitative image analysis as prerequisites to turning qualitative data into quantitative values. In our study, such quantitative data are expected to open the way toward a detailed view of interactions between nanoparticles and biological membranes. The method presented here holds many promises for future investigation of the potential of different nanoparticles to interact with lipid vesicles.

The applicability of lipid vesicles in studies with nanoparticles is that vesicles provide highly controllable and repeatable experimental conditions. Lipid vesicles are simple models for biological membranes. They are understood as flexible closed surfaces with dynamic shape transformations. In addition, such *in vitro* system is a cost effective mean for toxicological and pharmaceutical studies.

Even though our population based approach to identify properties of vesicles proved to be successful, our experimental and computer analysis algorithms still need improvement. In the future, our work will be focused on adapting and improving methods for automatic vesicle detection (circle and ellipse detection algorithms). Additionally, semi-supervised methods will be studied to combine the knowledge of experts with computer automated approach. When an expert segments a small subset of the images, the information obtained could be used to successfully identify similar objects in the rest of the images. Such semi-supervised methods could further improve the percentage of correctly identified vesicles and reduce amount of work required to manually segment images. Consequently, the same amount of expert's work would allow segmentation of even larger image sets and more relevant and precise calculations.

To sum up, the goal of our work is twofold. First, it would provide basic understanding of nanoparticle-membrane interactions and second, the information on biological potential of nanoparticles could be used as an additional, biological characteristic of nanoparticles apart their physicochemical properties.

ACKNOWLEDGMENT

We thank D. Erdogmus, D. Skočaj, B. Šter, and U. Lotrič for valuable discussions. We also thank S. Bolte for her technical assistance.

REFERENCES

[1] D. Drobne, A. Jemec and Z. P. Tkalec, "In vivo screening to determine hazards of nanoparticles: Nanosized TiO2," in *Environmental Pollution*, vol. 157, issue 4, pp.: 1157-1164, April 2009.

[2] S. Q. Li, R. R. Zhu and H. Zhu, "Nanotoxicity of TiO2 nanoparticles to erythrocyte in vitro," in *Food and Chemical Toxicology*, vol. 46, issue 12, pp. 3626-3631, December 2008.

[3] E. Salonen, S. Lin and M. L. Reid, "Real-Time Translocation of Fullerene Reveals Cell Contraction," in *SMALL*, vol. 4, issue 11, pp. 1986-1992, November 2008.

[4] W. Jian, B. Y. S. Kim and J. T. Rutka, " Nanoparticle-mediated cellular response is size-dependent," in *Nature Nanotechnology*, vol. 3, issue 3, pp. 145-150, March 2008.

[5] J. Z. Liu and A. J. Hopfinger, "Identification of possible sources of nanotoxicity from carbon nanotubes inserted into membrane bilayers using membrane interaction quantitative structure-activity relationship analysis," in *Chemical Research in Toxicology*, vol. 21, issue 2, pp. 459-466, February 2008.

[6] R. Lipowsky, M. Brinkmann, R. Dimova, C. Haluska, J. Kierfeld and J. Shillcock, "Wetting, budding, and fusion-morphological transitions of soft surfaces," in *Journal of Physics-Condensed Matter*, vol. 17, issue 31, Special Issue: Sp. Iss. SI, pp. S2885-S2902, August 2005.

[7] G. T. Linke, R. Lipowsky and T. Gruhn, "Free fluid vesicles are not exactly spherical," in *Physical Review E*, vol. 71, issue 5, Article Number: 051602, Part 1, May 2005.

[8] A. Imparato, J. C. Shillcock and R. Lipowsky, "Shape fluctuations and elastic properties of two-component bilayer membranes," in *Europhysics Letters*, vol. 69, issue: 4, pp. 650-656, February 2005.

[9] R. Lipowsky, "The Morphology of Lipid-Membranes," in *Current Opinion in Structural Biology*, vol. 5, issue 4, pp. 531-540, August 1995.

[10] J. Illingworth and J. Kittler, "The adaptive Hough Transform," in *IEEE Trans. Pattern Anal. Mach. Intell.*, pp. 690–698, 1986.

[11] T. Peng, A. Balijepalli, S. K. Gupta, T. LeBrun, "Algorithms for On-Line Monitoring of Micro Spheres in an Optical Tweezers-Based Assembly Cell," in *Journal of Comp. and Inf. Sci. in Eng.*, vol. 7, num. 4, pp. 330-338, 2007.