MICROFLUIDIC LIPOSOMES TARGETING HYPOXIA INDUCED TUMOR PROGRESSION

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ABSTRACT

This paper investigates the effectiveness of liposomes prepared by microfluidics on cellular uptake and trafficking in human breast cancer cells SKBR-3 and vascular protein sorting-associated protein 4B-knockdown SKBR-3 cells (SKBR-3 shVPS4B)[1]. We have utilized a formerly demonstrated microfluidic-based technique of liposome synthesis, by Jahn, et al., which presented new a avenue for producing liposome populations of tunable size with significantly decreased polydispersity compared to traditional bulk preparation methods [2]. Here, we have demonstrated the application of tunable size and monodispersity to facilitate specific targeting of drug delivery systems utilizing liposomes ranging in vesicle diameters of 40.6 nm to 276.6 nm.

KEYWORDS: Microfluidics, liposomes, breast cancer, endocytosis.

INTRODUCTION

Current liposome preparation techniques rely on bulk-scale synthesis and typically result in a limited range of size and uniformity [3]. Due to the size variability within a single population of liposomes, determination of an efficient size-based targeting mechanism remains a challenge. Size and size variability in liposomal delivery systems are important factors that can influence drug dosage, targeting and clearance [4]. The production of liposomes in microfluidics is an implementation of the alcohol-injection technique for liposome production within a microfluidic network, exploiting hydrodynamic focusing for controlled diffusive mixing of chemical species at the microscale to enable precise self-assembly of lipids into liposomes whose sizes may be dynamically adjusted by simply modifying the flow conditions within the device. This technique is a significant advancement in the preparation of liposomes, enabling formation of liposomes ranging from 30 nm to several hundred nanometers in diameter with minimal size variability in a given population [1][4].

The aim is to utilize monodisperse populations of liposomes to elucidate uptake mechanisms, as a function of liposome size and size distribution, for a unique tumor progressive shVPS4B cells in order to derive a more effective means of drug targeting and delivery. shVPS4B cells are particularly interesting due to their role in tumorogenesis and hypoxia. Hypoxia is a common feature in solid tumors and is an indicator of limited success of drug treatment. The down regulation of VPS4B in cells has shown to play a major role in hypoxia related migration within tumors [1]. The VPS4 protein complex participates in vesicular trafficking and autophagosome maturation in mammalian cells therefore any disruption of this pathway results in poor drug targeting outcomes [1].

EXPERIMENTAL

Microfluidic devices were fabricated using soft lithography techniques, where the microchannel patterns were made in SU-8 substrates and subsequently used as master molds for creating microchannels in PDMS. The PDMS pieces were bonded to glass via microwave-oven-generated plasma plasma, as described previously [2][5]. Lipid mixture was prepared with Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and dipalmitoylphosphatidylethanolamine-
polyethylene glycol 2000 (PEG2000-PE) (Avanti Polar Lipids) at 50/40/10 (mol%respectively) containing 1 wt % of a lipophilic membrane dye (1,1'-Diocatadecyl-3,3',3'-Tetramethylrhodaminecarbocyanine Perchlorate (DiI-C18)(Invitrogen). Lipid mix was re-hydrated in 10 mM phosphate buffered saline solution at pH 7.4. Liposomes used for cellular uptake experiments ranged from 40.6 nm to 276.6 nm in diameter (Table 1). The resulting liposomes were characterized using asymmetric flow field-flow fractionation (AF4) in-line with dynamic and static light scattering detectors for particle size characterization. These analyses showed the formation of nearly monodisperse liposomes.

The SKBR-3 and SKBR-3 shVPS4B cells were seeded in a 12-well plate (Corning, NY) and grown for 3 days - 4 days at 37°C, 95% relative humidity and 5% CO2. The cells were exposed to the liposomes for an hour before they were washed twice with a 1 ml solution of ice-cold Dulbecco’s phosphate buffered saline (DPBS). Cells were then incubated with trypsin for 5 min then transferred to microcentrifuge tubes, and centrifuged for 4 min at 1500 rpm. Cells were then washed in DPBS and finally fixed in 1% paraformaldehyde (in DPBS) solution and prepared for flow cytometry. The measurement was performed in a BD LSR flow cytometer (Becton Dickenson, Franklin Lakes, NJ) with filters appropriate for diI-C18. A 200 µL solution of 4% Trypan Blue was added to each sample and 10,000-20,000 events were taken per sample. Uptake analysis for the influence of liposome size on cell populations was determined by comparing the shift in means between each liposome size sample.

Intracellular trafficking of liposomes of different sizes within SKBR-3 and SKBR-3 shVPS4B cells was also investigated. Liposome co-localization with the endosomal or lysosomal compartments was determined in relation to the co-localization with antibodies for early endosomes (rabbit polyclonal early endosome antigen-1 (EEA-1)); and lysosome-associated membrane protein 1 (rabbit polyclonal lysosome antigen-1 (LAMP-1)); where the secondary antibody Alexa flour-488 goat anti-rabbit IgG (all from Molecular probes, Carlsbad, CA) was used. Images were acquired using a Nikon A1 Inverted confocal laser-scanning microscope (Nikon Instruments, Melville, NY). The extent of co-localization between the overlapping channels (where the red channel showed the liposomes and the green channel was either the endosomes or lysosomes) was determined using the Mander’s overlap coefficient (Moc), calculated directly through the Nikon Elements Software.

RESULTS AND DISCUSSION

SKBR-3 and SKBR-3 shVPS4B cells were incubated with liposomes of mean diameters ranging from 40.6 nm to 276.6 nm, with the number of liposomes introduced from each sample held constant (Fig 2). The values for total cellular uptake were determined by flow cytometry and normalized to liposome surface area. The resulting fluorescence data from flow cytometry indicated that cellular uptake was dependent on liposome size (Fig 2). The total cellular uptake for the smallest liposomes (40.6 nm) was significantly higher, almost 12-fold increase, than the largest liposomes (94.6 nm to 276.6 nm). 40.6 nm and 72.3 nm diameter liposome uptake was also much greater in the knockdown SKBR-3 cells than compared to the parent SKBR form. This trend was not seen in the much larger 94.6 nm to 276.6 nm liposomes. Therefore suggesting that the smaller size (40.6 nm to 72.3 nm) liposomes maybe a more favorable size for targeting these shVPS4B cells.

Intracellular trafficking of liposomes was observed in addition to determining the amount of uptake. Liposomes were expected to co-localize with the endosomes and lysosomes over time (Fig 3). However the aim here was to determine any differences in accumulation of the 40.6 nm liposomes with either the endosome (EEA-1) or lysosome (LAMP-1) compartments after 1 h of incubation at 37°C, within both types of cells. The co-localization these liposomes with the EEA-1 in the parent SKBR-3 cells was slightly higher than that observed with the LAMP-I (Fig 3). This suggests that after a 1 h incubation there may not be as many 40.6 nm liposomes that accumulate into both compartments within the cells. However, for the SKBR-3 shVPS4B cells, there appears to be slightly increased co-localization of the 40.6 nm liposomes with LAMP-1 compared to the parent SKBR-3 cells (Fig 3); implying that there could be an increased accumulation of 40.6 nm liposomes within lysosomal compartments after an hour of incubation. This may allow for higher percentage of liposomes (of small size) to accumulate in the lysosomes leading to a more effective drug release to the knockdown cells. However before accepting such observations, a much more detailed study of the internalizing and trafficking mechanisms for these unique shVPS4B cell line is required. Therefore our future efforts will involve a specific study detailing the endocytosis and internalization mechanisms influencing the size dependent uptake of liposomes by these unique knockdown cells and later use the effective size range to deliver the drug of interest.

![Figure 2: Uptake of liposomes by SKBR-3 and SKBR-3 shVPS4B cells incubated 60 min at 37°C with liposomes ranging from 40.6 nm to 276.6 nm in mean diameter containing DiI-C18 lipophilic dye. Fluorescence data was normalized to liposome diameter. Each cell population was incubated with 4 x 10^10 liposomes/mL. (Mean ± standard deviation (n=4), where *** indicates a significant difference (p<0.001) when comparing each column and all sizes)](image-url)
CONCLUSION

This study demonstrates cellular uptake studies with liposomes prepared by microfluidic flow focus-where liposome size and dispersity controlled. The ability to formulate populations of liposomes permits more detailed control over the intracellular fate of the drug carrier, thus aiding in more sophisticated design of liposomal drug delivery systems, it hopes to be beneficial for optimizing the particular drug delivery system of interest. Using the different sizes of liposomes produced through microfluidics we are able to identify the effective size range (between 40 nm to 70 nm diameter) for these unique hypoxia conditions mimicking cell line (SKBR-3 shVPS4B cells) and attempt to address the problems with drug resistance and tumor progression.

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DISCLAIMER

Certain commercial equipment, instruments, or materials are identified in this report in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

Figure 3 A) SKBR-3 cells with liposomes (red) & EEA1 (green). B) SKBR-3 shVPS4B cells with liposomes (red) & EEA1 (green). C) SKBR-3 cells with liposomes (red) & LAMP1 (green). D) SKBR-3 shVPS4B with liposomes (red) & LAMP1 (green). All using 40.6 nm mean diameter liposomes. Arrows indicate the regions co-localization between liposomes and endosome/lysosome pixels. Mander’s overlap coefficient estimates the extent of overlap between the red and green pixels (shown in the graph).