Nanoparticle Rigidity for Brain Tumor Cell Uptake

Chung-Fan Kuo, *Member, IEEE*, Fereshtehsadat Mirab, *Member, IEEE,* Dr. Mohammad Reza Abidian, *Member, IEEE,* Dr. Sheereen Majd, *Member, IEEE*

***Abstract*— Nanoparticles (NPs) have emerged as versatile and widely used platforms for a variety of biomedical applications. For delivery purposes, while some of NPs' physiochemical aspects such as size and shape have been extensively studied, their mechanical properties remain understudied. Recent studies have reported NPs' rigidity as a significant factor for their cell interactions and uptake. Here, we aim to study how NPs' rigidity affects their interactions with brain glioma tumor cells. To produce NPs with different rigidities, we encapsulate poly(ethylene glycol) diacrylate (PEGDA) of different volume ratios (0, 10, 30 v/v%) within the lumen of nanoliposomes and study the uptake of these NPs in a glioblastoma cell line U87. PEGDA with volume ratios of 10 and 30% were selected to provide a significant increase of the elastic modulus of the hydrogel (0.1 to 4 MPa) as determined by compression testing. Dynamic light scattering (DLS) and zeta potential measurements indicated that despite differences in their core formulation, all examined NPs had a similar size range (106 to 132 nm) and surface charge (-2.0 to -3.0 mV). Confocal microscopy revealed that all NP groups accumulated inside U87 cells, and flow cytometry data showed that liposomes with a gel core (10 and 30 v/v% PEGDA) had significantly higher cellular uptake (up to 9-fold), compared to liposomes with an aqueous core. Notably, we did not find any substantial difference between the uptake of liposomes with PEGDA core of 10 and 30% volume ratios.**

***Clinical Relevance*— By providing an insight into how NP rigidity influences glioma tumor cellular uptake, this work would enable development of more effective therapeutics for brain cancer.**

I. Introduction

NPs present a new frontier for a variety of biomedical applications [1]. This includes bioimaging [2], biosensors [3], vaccine development [4], and targeted drug delivery [5]. For drug delivery applications, much research effort has been focused on unraveling the influence of NP's physical and chemical properties such as particle size [6], shape [7], and surface charge [8] on their biological interactions and thus, their delivery performance. In recent years, particle rigidity has attracted attention as early studies have suggested that NP mechanics can be detrimental for their bio-distribution [9] and cellular interactions [10]. NPs' mechanical properties have been reported to influence their binding and internalization into tumor cells. For instance, Anselmo *et al.* reported that harder NPs, composed of PEGDA polymer with an elastic modulus of 3000 kPa, bound/internalized to 4T1 breast cancer cells significantly more than their softer (10 kPa) counterparts [9]. In another study, Sun *et al.* found that in case of PLGA (poly lactic-co-glycolic acid) NPs coated with lipid mono- or bi-layer, harder NPs (Young's modulus of 1.2 GPa) showed a higher level of association to HeLa cells compared to softer NPs (0.76 GPa) [11]. Interestingly, an opposite trend was reported in a different study for the cellular uptake of alginate gel-core liposomes by two breast cancer cell lines (MDA-MB 231 and MCF7), where softer NPs showed higher uptakes than the harder NPs [12]. These findings indicate that NP rigidity plays an important role in cancer cellular uptake.

Glioblastoma multiforme (GBM) is one of the most aggressive types of central nervous system tumors [13]. Although significant effort has been devoted to development of new therapeutics against these aggressive tumors [14-16], lack of effective carriers for delivery of these therapeutics remains a challenge in this field. Towards addressing this challenge, here we investigate the importance of NPs rigidity for their interactions with a human glioblastoma cell line, U87, *in vitro*. To this end, we focus on liposomes as they offer biocompatibility, bioavailability, and non-immunogenicity, and have had a great success in clinical applications among other carriers [17]. Incorporation of polymers into liposomes has been shown to modulate liposome's mechanical properties [18]. Here, to modulate the liposomal rigidity, we form PEGDA gel of different volume ratios inside the liposomes. This approach allows us to create NPs with similar size, shape, and surface properties, while varying their core rigidity. The resultant PEGDA-core liposomes were characterized using DLS and laser Doppler electrophoresis for particle size distribution and zeta potential, respectively. NP cellular uptake by U87 were investigated using confocal imaging and FACS analysis. This study may lead to development of improved delivery carriers for brain cancer in the future.

II. Materials and Methods

A. Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG2000-PE) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from Corning Inc. (Corning, NY). Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium, Gibco™ trypsin (2.5%), and 12-well flat bottom tissue culture plates were obtained from Fisher Scientific (Hampton, NH). Antibiotic/Antimycotic

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C-F. Kuo is with the Department of Biomedical Engineering, University of Houston, Houston, TX, 77204, USA (e-mail: ckuo9@central.uh.edu).

F. Mirab is with the Department of Biomedical Engineering, University of Houston, Houston, TX, 77204, USA (e-mail: fmirab@central.uh.edu).

M. R. Abidian is with the Department of Biomedical Engineering, University of Houston, Houston, TX, 77204, USA (e-mail: mabidian@uh.edu).

S. Majd is with the Department of Biomedical Engineering, University of Houston, Houston, TX, 77204, USA (Phone: 713-734-0866; e-mail: smajd9@uh.edu).

solution were from Gibco® BRL (Carlsbad, CA). Dow Sylgard 184 Silicone Elastometer Kit, the compounds of polydimethylsiloxane (PDMS), was purchased from Krayden (Denver, CO). 35 mm glass bottom dishes with 14 mm glass diameter were purchased from Matsunami (Osaka, Japan). Chloroform and centrifugal filter units with 10 kDa molecular weight cut-off were obtained from Millipore Sigma (Burlington, MA). Poly(ethylene glycol) diacrylate (PEGDA) with an average molecular weight of 700 Da, 2-hydroxy-2-methylpropiophenone (photo-initiator), and paraformaldehyde 95% were purchased from Sigma-Aldrich (St. Louis, MO). Vybrant™ CM-DiI Cell-Labeling Solution, NucblueTM fixed cell stain readyprobesTM reagent (DAPI) were procured from ThermoFisher Scientific (Waltham, MA). Naphtho 2,3-a pyrene (NAP) was purchased from Tokyo Chemical Industry Co., Ltd. (TCI) (Portland, OR). Fetal bovine serum (FBS) and TritonTM X-100 were supplied from VWR (Radnor, PA).

B. Evaluation of mechanical properties of PEGDA hydrogels

The elastic modulus of PEGDA hydrogels was determined by uniaxial compression testing (SwickRoell, Kennesaw, GA). Hydrogel solutions with concentrations of 10 and 30 v/v% PEGDA and 1 v/v% photo-initiator were poured into a PDMS mold with cylindrical wells and then exposed to UV light for 30 secs. The resultant cylindrical hydrogels were then removed from the mold for mechanical testing. The hydrogel specimen was placed between the loading plates of the instrument. The test was performed at a rate of 1 mm/min using a 50 N load cell. The stress (σ) was calculated by $\sigma=F/(\pi r^2)$, where F was the loading force, and r was the original radius of the specimen. The strain (ε) under compression was defined as the change in height (h) relative to the original height (h_0) of the free-standing specimen, $\varepsilon=(h_0-h)/h_0$. The slope of the compressive stress–strain curve within the strain range of 0.03-0.05 was defined as the elastic modulus of the hydrogel. At least five specimens were tested for each hydrogel concentration to calculate the average of the elastic moduli.

C. Fabrication of gel-core nanoliposomes

Previously, we have fabricated gel-core nano-liposomes by encapsulating PEGDA hydrogels into the lumen of the nano-liposomes [19]. Briefly, a lipid solution, containing DOPC and PEG2000-PE (95:5 molar ratio) in chloroform, with a total lipid concentration of 5 mM was dried by a rotary evaporator. The resultant lipid film was re-hydrated with 0, 10, and 30 v/v% PEGDA in DI water for 30 mins and bath sonicated for 10 secs to assure lipids were suspended in the solution. The suspension was extruded 9 passes through a 400 nm followed by 9 passes through a 100 nm porous polycarbonate membrane, using a mini-extruder (Avanti Polar Lipids, Inc, Alabaster, AL) to form PEGDA-encapsulating unilamellar vesicles. Next, the non-encapsulated PEGDA molecules were removed from the suspension via three rounds of centrifugation at 3500 rpm and resuspension in DI water using a cellulose centrifugal filter unit (10 kDa cut-off). Next, photo-initiator (2-hydroxy-2-methylpropiophenone) with a volume ratio of 1 v/v% was added into the suspension. It was well mixed, and then went through five freeze-thaw cycles in ice bath and water bath at 37 °C, each for five min. This process was utilized to enhance the inward transport of the photo-initiator across the liposomal membranes. The suspension was then exposed to UV light for 30 secs using a hand-held UV lamp (Blak-Ray, Upland, CA) with a light intensity of 16 mW/cm^2. The non-encapsulated photo-initiator molecules were removed via centrifugation as described above, and the particles were suspended in 1x DPBS. Lastly, NAP, as a fluorescent probe, was added into the particle suspension with a concentration at 1 mol% of total lipid composition, then stirred for 1.5 hours in a water bath at 37 °C. The resultant particle suspension was stored at 4 °C and used within two weeks.

D. Characterization of gel-core nano-liposomes

Size distribution and zeta potential of the gel-core nanoliposomes were assessed via DLS and laser Doppler electrophoresis, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), at room temperature. The gel-core nanoliposome suspensions were diluted 20 times in DPBS. Each measurement is the average of three instrument readings. At least three independent samples were used to calculate the average of particle size and zeta potential.

E. Confocal imaging of NP uptake by U87

Confluent U87, with a cell density of 500,000 cells/dish, were seeded in a 35 mm glass bottom dish for 48 hours prior to the experiment. After the initial incubation, the culture media contained 2.5×10^{11} NPs and was introduced to the cells, followed by a 6-hour incubation at 37°C. Once that incubation was complete, the NP containing media was removed and cells were washed with 1× DPBS twice. Afterwards, 1× DPBS that contained a dye, DiI, with a volume ratio of 1/1000 was added to each dish and incubated for 5 minutes at 37°C. Then an additional incubation was performed for 15 minutes at 4°C. The solutions were removed and the cells were washed with 1× DPBS twice. The cells were then fixed with 4% paraformaldehyde for 15 minutes, followed by permeabilizing with 0.1% Triton X-100 in PBS for 10 minutes. Both of these steps were done at room temperature. Cell membranes were washed by 1× DPBS twice after fixation and permeabilization. Cells were then incubated with a DAPI solution for 1 hour at room temperature, followed by washing with 1× DPBS twice for five minutes. Cells were imaged using an inverted microscope (Zeiss LSM800, Germany). NAP-labeled NPs, cell membrane, cytoplasm, and nucleus were imaged using excitation wavelength of 305, 549 and 360 nm and emission wavelength of 470, 565, and 460 nm, respectively.

F. Quantitative analysis of cellular uptake by FACS

Confluent U87 cells were seeded into 12-well plates with a seeding density of 250,000 cells/well. After culturing for 48 hours, media was removed. All types of fluorescent

nanoparticles with a particle number of 2.5×10^{11} diluted in fresh media were introduced to cells and incubated for 1, 3, and 9 hours. After incubation, cells were washed with DPBS, trypsinized, and then harvested in 1× DPBS. The fluorescent intensity of NAP-NPs uptaken by cells was determined by FACS analysis using flow cytometry (Guava easyCyte 8HT, Millipore, USA). Fluorescence intensity of the NPs at all the time points were further normalized to the fluorescence intensity of the liposomes at 1 hour to determine the changes in cellular uptake influenced by time and elasticity.

G. *Statistical analysis*

All data is presented as mean ± STD, collected from at least three independent samples, and was analyzed for statistical significance by student t-test using Prism software (San Diego, CA). Differences were considered statistically significant for P values < 0.05.

III. Results and Discussion

A. *Elastic modulus of PEGDA hydrogels*

Before fabricating the gel-core liposomes, we evaluated the change of elastic modulus of PEDGA hydrogels with different polymer volume ratios, using uniaxial compression testing. The measurements revealed that increasing the PEGDA volume percentages (from 10 to 30 v/v%) while keeping the photo-initiator content (1 v/v%) unchanged, led to a significant increase in PEGDA hydrogel's Young's modulus from 0.1 to 4 MPa (Fig. 1A). This increase is presumably due to a denser polymer network in the 30% PEGDA gel compared to 10%, resulting in higher cross-linking percentage. We thus proceeded to fabrication of PEGDA-core liposomes using 10 and 30% v/v% PEGDA ratios as medium and hard NPs, respectively. Aqueous core liposomes (without PEDGA cores) were used as the soft NPs in this study (Fig. 1B).

B. *Characterization of PEGDA gel-core liposomes*

NP rigidity is often modulated by varying material composition that can result in un-intended changes in other NP properties such as surface chemistry [9], presenting a challenge for studying the sole effect of NP rigidity. To overcome this challenge, we take a simple approach of utilizing nanoliposomes with a PEGDA core to modulate the particle rigidity without changing other NP properties, such as size and surface chemistry. We previously applied this approach to create PEGDA-core liposomes and confirmed the formation of PEGDA within liposomes using DLS and scanning electron microscopy (SEM) [19]. Here, we varied the PEGDA volume ratio in liposomes and to assess the effect of this change on other NP properties, we evaluated their size distribution and zeta potential. As demonstrated in Fig 2A, DLS measurements revealed that the resultant NPs, containing different PEGDA volume ratios, had comparable hydrodynamic diameters within 106 to 132 nm. These data further showed that all three NP groups had a relatively narrow size distribution with an average PDI smaller than 0.2. As shown in Fig 2B, the zeta potential measurements also showed slight variations between different NP groups (-2.0 to -3.0 mV) as these liposomes had identical lipid composition. These results demonstrate that our liposomal NPs had a similar size distribution and surface charge despite various core formulations and rigidities.

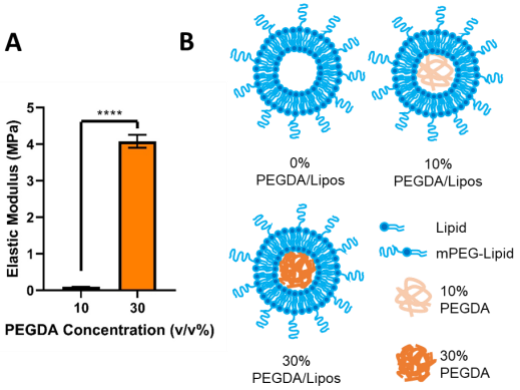

Figure 1. Characterization of PEGDA hydrogels. (A) Elastic modulus of 10 and 30 PEGDA bulk gels. n=5, ****< 0.0001. (B) The schematics of PEGDA gel-core liposomes (PEGDA/lipos) with different ratio of PEGDA concentrations.

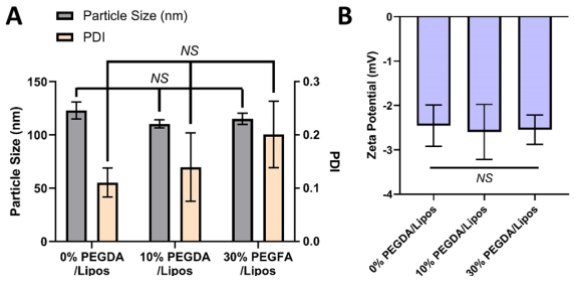

Figure 2. Characterization of PEGDA gel-core liposomes. DLS results of particle size and PDI (A), and zeta potential (B) of 0, 10, and 30% of PEGDA gel inside the liposomes. n=3, NS: non-significant.

C. *Cellular uptake of the gel-core liposomes by human glioblastoma U87 cells*

In order to study how NP rigidity influences cellular uptake in human glioblastoma U87 cells, we first performed confocal imaging of U87 to visualize the internalization of NPs. Cells were incubated with NPs labeled with NAP (green fluorescence) for 6 hours. The cytoplasm and nucleus of the cells were labeled with DiI (red) and DAPI (blue), respectively. As shown in Fig. 3A, all types of the NPs could be observed within the cytoplasm (yellow).

We further investigated this uptake using FACS analysis. As summarized in Fig. 3B, we observed a time-dependent increase in the uptake of all NPs and notably, both medium NPs (10% PEGDA-core) and hard NPs (30% PEGDA-core) showed a significant increase in uptake, compared to the soft NPs without PEGDA gel core at all examined time points. Specifically, medium NPs had approximately 3-, 6-, and 10-fold increase in uptake compared to aqueous-core liposomes at 1, 3, and 9-hour incubation times, respectively. Similarly, hard NPs showed approximately 3-, 6-, and 9-fold increased uptake compared to aqueous-core liposomes during the same incubation period (Fig. 3B). These results suggest that U87 cells are more partial to uptake NPs with higher rigidity than their softer counterparts. This finding is also in agreement with previous studies that have shown higher cellular uptake for harder NPs in cancerous cells compared to soft NPs [9, 11]. The lower uptake tendency of soft NPs may be attributed

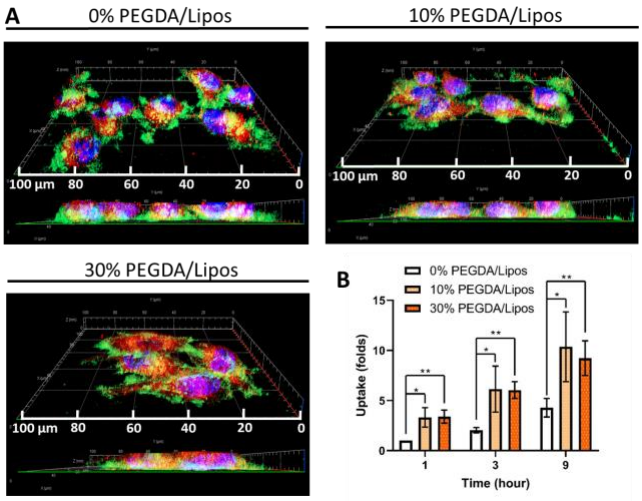

Figure 3. Qualitative and quantitative analysis of NP cellular uptake by glioblastoma cells U87, using (A) confocal 3D images and (B) FACS analysis. n=3, *<0.05, **<0.001.

to its deformability, which translates to higher level of energy and time required to wrap the plasma membrane around NPs during the uptake process [11, 20]. Interestingly, as illustrated in Fig 3B, we found no significant difference in cellular uptake among medium and hard NPs at the examined time points. This result may be explained by the cells not being able to sense the difference in the rigidity between 10% and 30% PEGDA gel-core liposomes, resulting in similar uptake levels of those NPs (Fig. 3B). In addition, the rigidity of NPs has been shown to impact their mobility through tumor extracellular matrix [21], and may thus, allow for the design of better nanocarriers in future.

IV. Conclusion

In this study, we prepared three groups of NPs with various rigidities by altering the volume percentage of the PEGDA in the lumen of nanoliposomes. Our results demonstrated that despite the differences in the core of these liposomes, they had a similar particle size distribution as well as zeta potential. Using confocal microscopy, we confirmed that all groups of NPs were up-taken by the U87 cells within 6 hours. Through further investigation using flow cytometry, we found that PEGDA-core liposomes, with both 10 and 30% PEGDA core, showed a significantly higher level of uptake (up to 9-fold) in glioma U87 cells, compared to traditional aqueous-core liposomes. However, the U87 uptake of liposomes with 10 and 30% PEGDA cores was comparable across all the examined time-points and did not show any significant difference. Thus, further investigation into differences among liposomes with polymeric cores may be needed. Together, these results highlight the role of NP mechanical properties in their interactions with brain tumor cells. The findings of this work may provide new approaches to improve the brain cancer therapeutic.

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