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Authors Melis Emanet, Özlem Şen and Mustafa Culha

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ORCID® iDs Özlem Şen - https://orcid.org/0000-0002-6237-0264; Mustafa Culha - https://orcid.org/0000-0002-3844-5190

Transferrin-mediated glioblastoma cell targeting of hexagonal boron nitrides

Melis Emanet[1], Özlem Şen[1] and Mustafa Çulha[*]

Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Ataşehir, Istanbul 34755, Turkey

***Corresponding Author**

Mustafa Çulha,

Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Ataşehir, Istanbul 34755, Turkey

Tel: +90 (216) 578 1587

Fax: +90 (216) 578 0829

E mail: mculha@yeditepe.edu.tr

[1]These authors have equally contributed to this work.

Abstract: Hexagonal boron nitrides (hBNs) are promising nanomaterials with their high boron content, non-toxic nature in inactive form, high chemical stability and mechanical strength. However, their hydrophobic nature limits their use in biomedical applications. Therefore, the hBNs have been functionalized with DSPE-PEG-NH_2 to increase their colloidal stability and circulation time in bloodstream, as well as to provide active sites on their surface for further functionalization with tumor targeting agents. Then, further functionalization of the DSPE-PEG-hBNs with transferrin was performed for selective targeting of transferrin receptors overexpressed by brain tumor cells. Then, the cellular interaction and biocompatibility of the structure was investigated on glioblastoma multiform (U87MG) cancer cells. The cellular investigations showed that transferrin functionalization of the DSPE-PEG-hBNs increased their uptake by glioblastoma cancer cells, and decreased cell viability due to the enhanced cellular internalization. Based on the data, the TrF-DSPE-PEG-hBNs are promising agents to evaluate them as drug carrying and targeting applications.

Keywords: cytotoxicity, drug delivery, DSPE-PEG, hexagonal boron nitride, transferrin

1. INTRODUCTION

In recent years, engineered nanoparticles have gained significant interest in diagnosis and treatment of diseases. Thanks to their small and unique sizes caused to high surface to mass ratio that provides excellent ability of functionalization with other structures such as drug and targeting agents by binding covalently or non-covalent interactions. Drug and targeting agent binding or absorption on the surface of the nanoparticles not only increase their cellular internalization capacity but also regulates the intracellular fate of the structures [1-3]. Therefore, a large number of varied biological compounds including proteins, polymers, carbohydrates, phospholipids and liposomes under investigation for their effects on nanoparticle delivery to the desired cells and tissues depend on the aim of the study [4]. As commonly used polymeric surface modifiers, poly(ethylene glycol) (PEG), provides prolonged circulation of nanoparticles in circulatory system by preventing recognition by mononuclear phagocytic cells as a foreign structure and also increase the endosomal, cytosolic and nuclear intracellular accumulation [5]. Besides, entrapment of nanoparticles with liposomes or functionalization with phospholipids in nanosized forms have advantages of being small, flexible and biocompatible structures that able to pass through the vein endothelial cells without causing any cloths [6]. Owing to the different physico-chemical characteristic behaviours of nanosized particles from their micro sized counterparts, they have changed body distribution performance and high ability to pass through barriers in the body. Therefore, their surface chemistry must be well developed with functionalization in case of toxic nature of nanoparticles as well as prevent agglomeration of especially hydrophobic nanoparticles that provides increased colloidal stability in physiological conditions [7].

Hexagonal boron nitrides (hBNs) are structural analogues of graphene, have strong intra-layer covalent bonding and weak inter-layer van der Waals interaction [8]. hBNs can be potentially used in many fields including pharmaceutical sector, cosmetics, chemistry and high-

temperature technologies due to their biocompatibility, high chemical and thermal stability, excellent mec mechanical strength and electrical insulation properties [9,10]. Besides the excellent properties, high boron content of the hBNs makes them suitable active agents for radiotherapy applications such as boron neutron capture therapy [11]. Therefore, their effective targeting into the cancerous cells plays very important role in their therapeutic investigations.

In this study, hBNs were first non-covalently interacted with PEG-NH_2 including phospholipid (DSPE- PEG-NH_2) to increase their colloidal stability in aqueous environment by increasing hydrophilicity and providing active ends for further functionaliza-tion. The interaction occurs between the hBN side walls and the phospholipid tails of the DSPE-PEG-NH_2. The characterization of DSPE-PEG-NH_2 interacted hBNs (DSPE-PEG-hBNs) was performed using UV/Vis spectroscopy and thermogravimetric analysis (TGA). Besides, their colloidal stability was analysed with dynamic light scattering (DLS). Furthermore, the DSPE-PEG-hBNs were covalently functionalized with transferrin (TfR-DSPE-PEG-hBNs) exploiting the NHS groups of the DSPE-PEG-hBNs and the carboxyl side groups of the protein. The transferrin binding efficiency around DSPE-PEG-hBNs was further investigated with bicinchoninic acid (BCA) measurement. The biocompatibility and cellular uptake of the structure were tested on glioblastoma multiforme cells (U87MG) as a malignant brain cancer cell type.

2. RESULTS AND DISCUSSION

2.1. hBN Synthesis and Characterizations

The synthesized hBNs were characterized using SEM as an imaging technique and with FT-IR as a spectroscopic technique. From the SEM image, the average lateral size dimensions of hBNs is found to be about 50 nm as shown in Fig. 1A. In the FT-IR spectrum, the peaks

originating from B-N vibrations at around 1360 and 820 cm^{-1} indicate a successful synthesis of hBNs as shown in Fig. 1B.

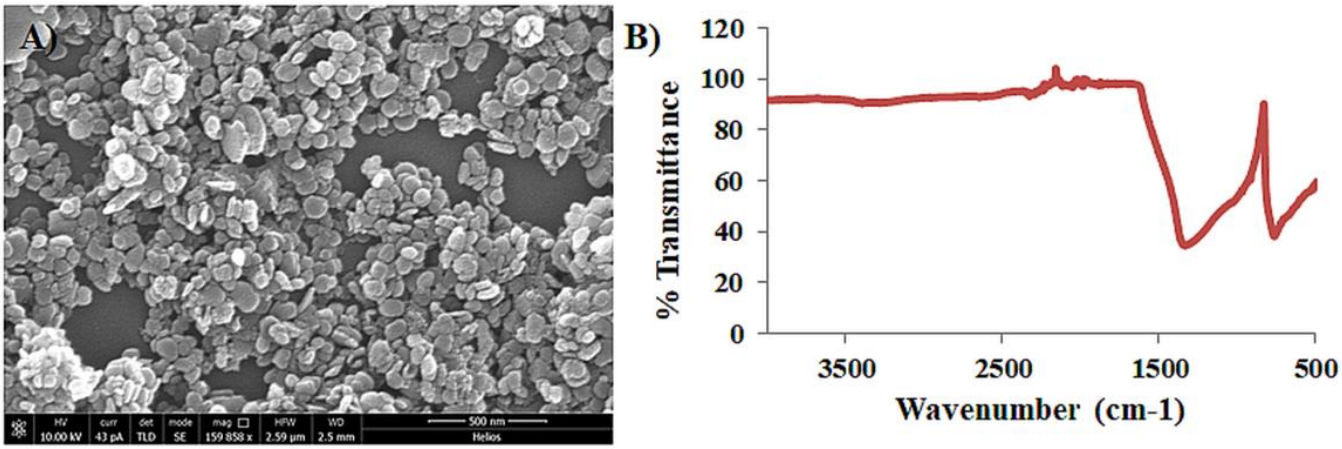

Figure 1*. SEM image (A) and FT-IR measurement result (B) of synthesized hBNs.*

2.2.Characterization of DSPE-PEG-hBNs

The hBNs were interacted with DSPE-PEG-NH_2 molecules that have two hydrophobic fatty acid chains and a hydrophilic PEG structure that includes also -NH_2 end for further covalent func-tionalization. The interaction occurred between the sidewalls of the hBNs and the hydrophobic fatty acid chains of the DSPE-PEG-NH_2 molecules. The bare hBNs, DSPE-PEG-NH_2 and DSPE-PEG-hBNs were comparatively characterized using UV/Vis spectroscopy as shown in Fig. 2A. The UV/Vis spectra of DSPE-PEG-hBNs shows characteristic absorption peak of the hBNs, originated from B-N bonds, around 210 nm wavelength, while the DSPE-PEG-NH_2 specific absorption peaks were observed around 200 and 280 nm.

The DSPE-PEG-NH_2 interaction with hBNs was moreover quantified using TGA analysis as shown in Fig. 2B. Due to the high thermal stability of the hBNs, the 12% weight lose of the DSPE-PEG-hBNs around 240-400^oC shows the interaction yield of DSPE-PEG-NH_2 molecules.

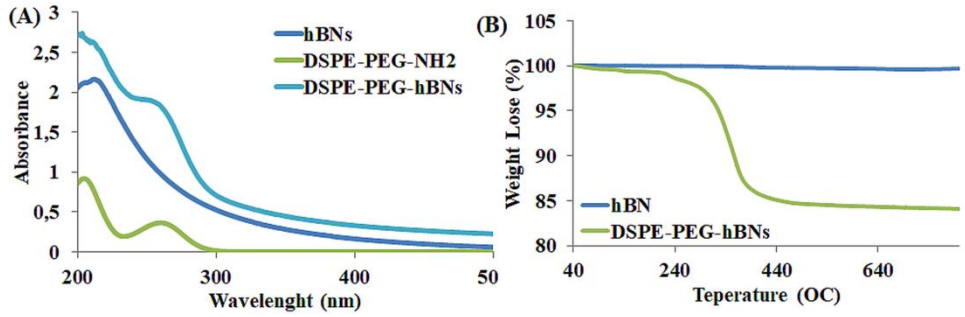

Figure 2. *UV/Vis (A) and TGA (B) analysis of hBNs and DSPE-PEG-hBNs.*

2.3.Colloidal stability of DSPE-PEG-hBNs

The hydrophobic nature of the hBNs limits their use in biological applications due to the poor colloidal stability in aqueous media. Thus, the main propose of the DSPE-PEG-NH_2 interaction was providing hydrophilic surface around the hBNs and increasing their colloidal stability. The colloidal stability of hBNs and DSPE-PEG-hBNs was comparatively investigated with DLS by measuring their particle size distribution at different timepoints (0, 1 and 24 h) following dispersion (Fig. 3). The size distribution of DSPE-PEG-hBNs was found to be between 120 and 230 nm and their average size was approximately 164 nm, while the hBNs size distribution was between 120 and 290 and their average size was approximately 190 nm just after the interaction (Fig. 3A). Following the interaction at 1 h the size distribution of the DSPE-PEG-hBNs was found to be between 100 and 290 nm and their average size approximately was 190 nm, while hBNs size distribution was between 60 and 540 and average size approximately 255 nm, as shown in Fig. 3B. After 24 h, the size distribution of the DSPE-PEG-hBNs was found to be between 100 and 390 nm and their average size was approximately 220 nm, while hBNs size distribution was between 60 and 660 and average size was approximately 260 nm as shown in Fig. 3C. It has been observed that the size distribution of DSPE-PEG-hBNs is narrower at each time points of measurement

as compared to the hBNs, indicating the higher colloidal stability of DSPE-PEG-hBNs in aqueous environment with respect to the bare form of hBNs. Moreover, the lower average size of the DSPE-PEG-hBNs comparing to the hBNs shows their higher dispersion capacity.

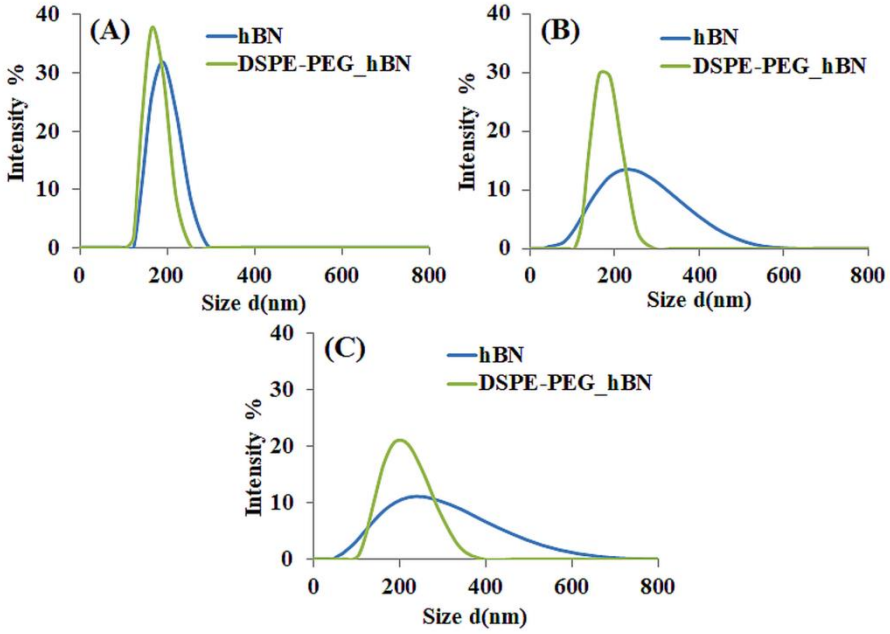

Figure 3. *Size distribution of the hBNs and DSPE-PEG-hBNs at 0 (A), 1 (B) and 24 h (C) after the dispersion.*

2.4.Characterization of TfR-DSPE-PEG-hBNs

Generally, NH_2 ends of the molecules are used as activating agent of carboxylic acid groups of molecules [13]. In this study, they were used as active end for the modification of the DSPE-PEG-hBNs with the carboxylic acid groups of the amino acids belonging to the transferrin protein. Transferrin binding efficiency over the DSPE-PEG-hBNs was investigated using BCA protein quantification tests, and results indicated the 1% binding efficiency of transferrin.

2.5.Cellular uptake and biocompatibility of TfR-DSPE-PEG-hBNs

Glioblastoma multiforme cells (U87MG) were chosen as a model brain cancer cells with overexpressed transferrin receptors on the cell surface [14]. The cellular uptake of hBNs, DSPE-PEG-hBNs, and TfR/DSPE-PEG/hBNs was comparatively analyzed by measuring the side scattering of the cells using flow cytometer as shown in Fig. 4A. The results indicate that the hBN uptake of the cells regularly increased up to 28% while the DSPE-PEG-hBNs were increased to the 25% at the 24 h of incubation. The TfR-DSPE-PEG-hBNs uptake is also increased regularly up to 35% at the 24 h of incubation. Moreover, the uptake of the hBNs and DSPE-PEG-hBNs continued to increase up to 34% and 32% while the uptake of the TfR-DSPE-PEG-hBNs decreased to the 32% at the 48h of incubation. The data indicate a successful cellular uptake of the bare hBNs by penetrating the cells via passive diffusion. The results also explain the decreased cellular uptake of the DSPE-PEG-hBNs due to the increased hydrophilic nature of the structures with the DSPE-PEG-NH_2. Moreover, significantly increased cellular uptake of the Tfr-DSPE-PEG-hBNs up to 24h of incubation indicates increased uptake of the structure due to the transferrin functionalization. Additionally, the toxic nature of the overdose of Tfr-DSPE-PEG-hBNs decreases the cell viability. Thus, it is estimated that the cellular uptake of the TfR-DSPE-PEG-hBNs decreased at the 48 h of incubation.

Then, the biocompatibility of the hBNs, DSPE, DSPE-PEG-hBNs, transferrin and TfR-DSPE-PEG-hBNs were investigated using WST-1 colorimetric assay as shown in Fig. 4B. According to the results, while the viability of the hBN exposed cells increased to the 105%, the DSPE-PEG-NH_2 and DSPE-PEG-hBNs exposed cell viability decreased to the 87% and 84% at high concentrations (100 µg/mL). However, the TfR/DSPE-PEG/hBNs exposed cell viability decreased to the 49% at high concentrations (100 µg/mL), because of the toxic nature of the TfR/DSPE-PEG/hBNs at high concentrations.

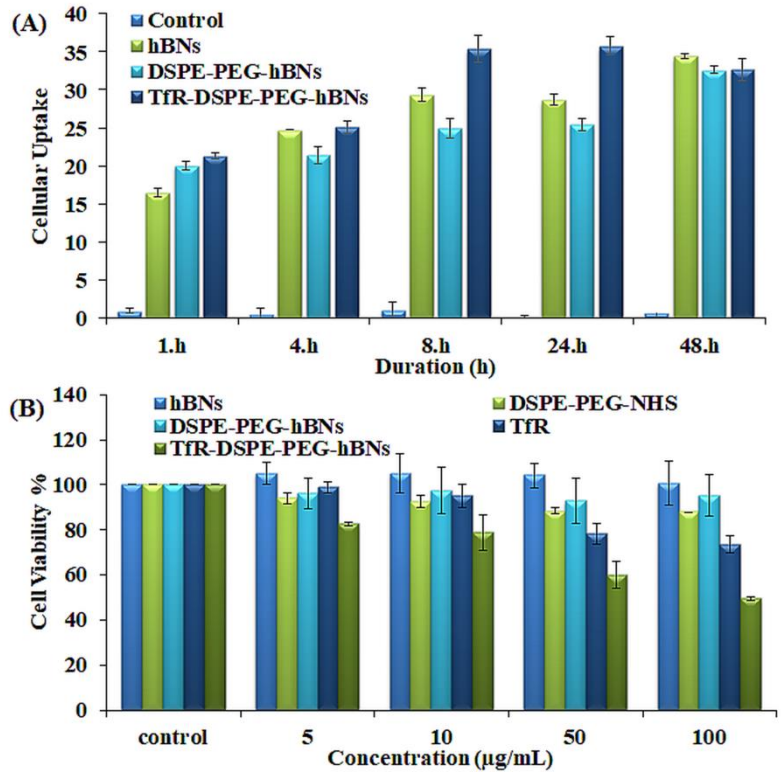

Figure 4. *Cellular uptake (A) and biocompatibility (B) evaluations of hBNs, DSPE, DSPE-PEG-hBNs, transferrin and TfR-DSPE-PEG-hBNs.*

Confocal microscopy images were acquired to observe the inter-nalization of free FITC, TfR-FITC-DSPE-PEG-hBNs (10 µg/mL) and TfR-FITC-DSPE-PEG-hBNs (100 µg/mL) by the cells after 4 h of incubation. The fluorescence originating from FITC allowed tracking uptake of the TfR-FITC-DSPE-PEG-hBNs into the glioblastoma cells as shown in Fig. 5. As seen in the figure, the free FITC and TfR-FITC-DSPE-PEG-hBNs structures was successfully internalized into the cells and separated in the cytosol. It is also seen that the both concentration of TfR-FITC-DSPE-PEG-hBN exposed cells have apoptotic bodies in the nucleus while the 100 µg/mL of TfR-FITC-DSPE-PEG-hBNs exposed cells lost their cellular integrity. All the cellular experiments clearly indicate the TfR-DSPE-PEG-hBNs have a

superior cellular uptake capacity that is the reason of the strong cell viability decrease of glioblastoma cancer cells even in their inactive form.

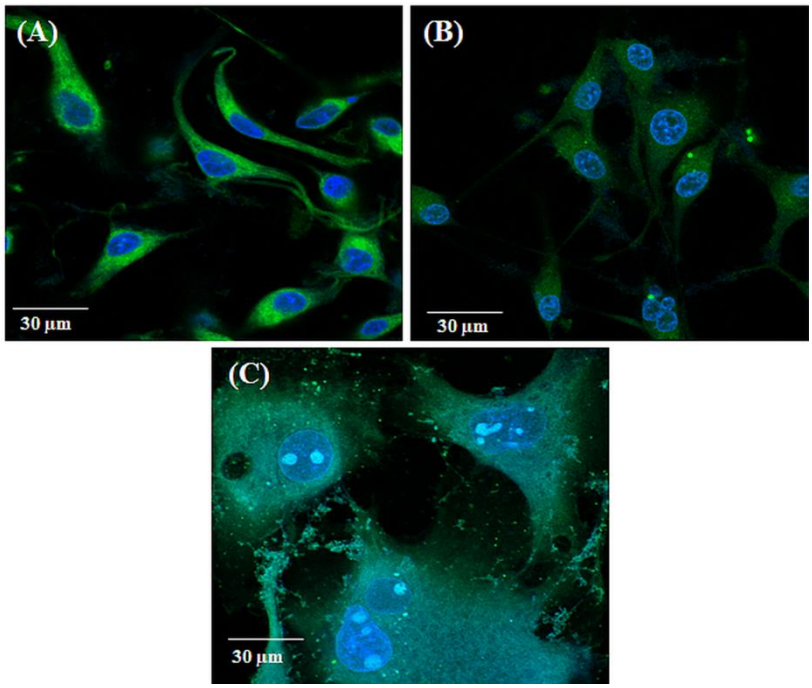

Figure 5. *Confocal images of the glioblastoma cells exposed to the FITC (A), TfR-FITC-DSPE-PEG-hBNs (10 µg/mL) (B) and TfR-FITC-DSPE-PEG-hBNs (100 µg/mL) (C).*

3. CONCLUSIONS

In this study, the synthesized hBNs were intended to be generated as a boron-including active agent. Since the hydrophobic nature of the hBNs, they were first interacted with DSPE-PEG-NH_2 molecules to increase solubility in aqueous media and to have active ends for further functionalization. Then, the transferrin protein as a cancer cell targeting agent was covalently bound to the active ends of the DSPE-PEG-hBNs. The comparatively performed char-acterization studies indicated an optimal DSPE-PEG-NH_2 interac-tion was succeeded. Promisingly, the transferrin functionalization of the hBNs significantly increased their cellular uptake and caused overdose-dependent cell viability decreasing in glioblastoma cancer cells.

Thus, the transferrin functionalized hBNs are suggested as possible boron source active agents.

4. EXPERIMENTAL

4.1.Synthesis and Characterization of hBNs

The synthesis of hBNs was performed following a method previously described [12]. First, 2 g of boric acid were suspended in 3 mL ammonia solution. The mixture was transferred onto the silicon carbide boat and dried by evaporation on heating system at 100°C for 20 min. The silicon carbide boat was then placed into the Protherm, Furnaces PTF 14/50/450. The hBN synthesis was performed under NH_3 gas flow for 2 h at 1300°C, the heating rate was set at 10°C/min. Following the synthesis, the hBNs were removed from furnace around 550°C, scratched from top of the silicon carbide boats, and kept in dried conditions at room temperature. The characterization of the hBNs was performed with scanning electron microscopy (SEM) (Carl Zeiss Evo 40) as an imaging technique and FT-IR (Thermo NICOLET IS50 Spectrometer) as a spectroscopic technique.

4.2.hBN Interaction with DSPE-PEG-NH_2 and Characterization

The hBNs and DSPE-PEG-NH_2 interaction was optimized using several concentration ratios of components and varied sonication durations (data not shown). In the optimized protocol, the hBNs were ultrasonicated for 2 min for an efficient dispersion. Then, the hBNs and DSPE-PEG-NH_2 were ultrasonicated for 1 min to allow their interaction. Following this process, intensive washing proce-dure was applied to remove the weakly bound DSPE-PEG-NH_2 molecules from the hBNs. The characterization of the DSPE-PEG-hBNs was performed with UV/Vis spectroscopy (Perkin Elmer Lambda 25 UV-Vis spectrometer); the measurements were per-formed three times. Furthermore, the DSPE-PEG-hBNs interaction

yield was analyzed using TGA (Mettler Toledo TGA/SDTA 851 instrument) following a drying process in vacuum oven at 60ºC for 24 h to remove water molecules in the sample. The samples were analyzed with TGA under 20 mL/min N_2 gas flow. The temperature was increased up to 700ºC at a rate of 10ºC/min.

4.3. Colloidal Stability of DSPE-PEG-hBNs

The colloidal stability analysis of hBNs and DSPE-PEG-hBNs was performed comparatively by monitoring the size distribution of the samples in borate buffer. According to the DSPE-PEG-hBNs inter-action yield, the concentration of the hBNs and DSPE-PEG-hBNs were fixed as 100 µg/mL and 112 µg/mL, respectively. Following the interaction process, samples were assessed with DLS at several times (0, 1 and 24 h) to evaluate their time-dependent colloidal stability.

4.4. DSPE-PEG-hBN Functionalization with Transerrin and Characterization

In the next study, NH_2 groups in DSPE-PEG-NH_2 structure were used as active end for the modification of the DSPE-PEG-hBNs with the carboxylic acid groups of the amino acids belonging to the transferrin protein. 112 µg DSPE-PEG-hBN and 100 µg transferrin were incubated by shaking overnight in borate buffer (pH 9) at 4ºC. Then, the samples were washed three times with centrifuga-tion and free transferrin molecules were removed from the reaction environment. The interaction yield of TfR-DSPE-PEG-hBNs was performed with BCA assay. In the BCA measurements, several concentrations of transferrin (between 0-250 µg/mL) were prepared as standard solutions to reveal the interacted transferrin concentration. 25 µL of hBN, DSPE-PEG-hBN, TfR-DSPE-PEG-hBN samples and standard solutions were added to 2 mL of BCA working reagents in 96 well-plates and incubated for 30 min at 37ºC. Since the method relies on reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium, the

reduction of the Cu^{2+} was measured using an unique reagent containing bicinchoninic acid that can be detected with sensitive and selective absorption of light at 562 nm.

4.5. Cellular Experiments of hBNs, DSPE-PEG-hBNs and TrF-DSPE-PEG-hBNs

4.5.1. Cell Culture

U87MG glioblastoma multiforme cell line was utilized to assess the cellular uptake and cytotoxicity of hBNs, DSPE-PEG-hBNs and TfR-DSPE-PEG-hBNs. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin/ampicillin (PSA) antibiotics and supplemented with 1% L-glutamine in addition to other components. The cells were incubated in water-jacketed incubator in a 5% CO_2, 95% air atmosphere at 37°C.

4.5.2. Cellular Interaction of hBNs, DSPE-PEG-hBNs and TrF-DSPE-PEG-hBNs

The cellular interaction of hBNs, DSPE-PEG-hBNs and TfR-DSPE-PEG-hBNs by U87MG cells was investigated using flow cytometer. In the study, $6x10^4$ U87MG cells were seeded in 24-well plates and incubated for 24 h. Then the medium of cells was replaced with the medium that includes a single concentration (50 µg/mL) of hBNs, DSPE-PEG-hBNs, or TfR-DSPE-PEG-hBNs. They were incubated at several incubation times (1, 4, 8, 24 and 48 h). Thereafter, cells were removed from plate following a trypsin treatment and dispersed in phosphate buffer saline (PBS) for flow cytometry measurements. Based on the increased side scattering of the cells due to the uptake of hBNs, DSPE-PEG-hBNs and TfR-DSPE-PEG-hBNs they were analysed by flow cytometer.

4.5.3. Biocompatibility of hBNs, DSPE-PEG-hBNs and TrF-DSPE-PEG-hBNs

Biocompatibility of hBNs, DSPE-PEG-hBNs and TfR-DSPE-PEG-hBNs was quantified with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-

1) colori-metric assay. First, 6x103 U87MG cells/well were seeded in 96-well plates and incubated for 24 h. Then, the medium of cells was replaced with the hBNs at several concentrations (5, 10, 50 and 100 µg/mL) and their equal concentration of DSPE-PEG-hBNs and TfR-DSPE-PEG-hBNs. Following 24 h of incubation, the culture media was replaced with fresh culture media containing WST-1 reagent at 1:10 ratio and further incubated for 1 h. The percentage of living cells is calculated by measuring absorbance of formed formazan salts at 450 nm with a microplate reader.

4.5.4. Confocal Microscopy Imaging of TrF-DSPE-PEG-hBNs treated cells

The cellular uptake of TfR-DSPE-PEG-hBNs was observed by using FITC-labeled DSPE-PEG-NH_2 structures by confocal mi-croscopy. In this study, the DSPE-PEG-NH_2 molecules were cova-lently modified with FITC by shaking overnight in borate buffer (pH 9) at 4ºC. The free FITC molecules were removed from the inter-action solution by washing three times using filter-based centrifu-gation process. Then, 1 mg of hBNs was incubated with 249 µg of DSPE-PEG-FITC and DSPE-PEG-NH_2 at a ratio of 1:4. Finally, DSPE-PEG-FITC-hBNs were modified with transferrin by shaking overnight in borate buffer (pH 9) at 4ºC. Then, he samples were washed three times with centrifugation and free transferrin mole-cules were removed from reaction environment. Next, $2x10^5$ U87MG cells/well were seeded on cover slips at 6-well plate and incubated overnight. Then, the media of the cells were replaced with the 10 µg/mL of TfR-FITC-DSPE-PEG-hBN, and 100 µg/mL of TfR-FITC-DSPE-PEG-hBN including fresh media and incubated for 4 h. Following the incubation, cells were fixed with 4% para-formaldehyde at 4ºC for 30 min and washed with PBS for 5 min three times. The samples were examined with a Zeiss LSM 700 confocal laser-scanning microscope.

5. ACKNOWLEDGEMENTS

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