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Synthesis of Supramolecular Ionic Liquid Grafted Three-Dimensional Nitrogen-Doped Graphene as a Modified Cationic Polymer

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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In this report, supramolecular ionic liquid supported on three-dimensional nitrogen-doped graphene-based frameworks with folate (SIL-g-3D-(N)GFs-folate) was successfully synthesized and characterized. The super dispersion of SIL-g-3D-(N)GFs in water and also high tendency to the nucleic acid and negatively charged cell membrane probably makes it an ideal candidate for biological purposes.

Gene therapy, which aims to replace defective genes inside cells in order to treat diseases, holds promise for treating cancers because of the various pathways it can exploit.[1] For instance, one could alter a patient's immune system to recognize cancerous cells, replace the malfunctioning genes needed for proper cell regulation and function, or enhance the sensitivity of cancer cells to chemotherapy.[2] Modified viruses have been used for gene therapy, but their use is limited by safety concerns and large-scale production difficulties.[3] Non-viral materials have the potential to overcome these concerns,[4, 5] but they are currently limited by inefficient delivery and nonspecific binding.[6] Cationic polymers are able to condense negatively charged nucleic acids into positively charged polyplexes amenable to translocation across negatively charged cell membranes.[7-9] After cellular entry via endocytosis, the polymer/plasmid complexes can undergo dissociation via endosomal escape to release nucleic acid into the nucleus for gene expression.[10] Such processes can also allow different types of released RNA to execute their biological functions inside cells[11, 1211, 1211, 1211, 1211, 1211, 1211, 12 11, 12] A great number of polycations, including polyethylenimine (PEI), poly((2-dimethyl amino)ethyl methacrylate) (PDMAEMA), poly(l-lysine) (PLL) and polyamidoamine (PAMAM), have been reported to be capable of delivering genes.[13-16] Recently, due to the importance of poly ionic liquids, several pioneer groups published reviews about this hot topic.[17-19] The previous reports establish that many enzymes and whole cells can keep their activity in ILs.[20, 21] Imidazolium-based ILs due to their cationic nature have high binding ability to the nucleic acid and negatively charged cell membrane and can effectively improve cellular uptake via electrostatic interaction. Additionally, by delocalizing their positive charge into the heterocyclic ring, the imidazolium salts can spread their charge which might decrease the cytotoxic effect associated with the cationic nature of the vectors.[22] Poly ionic liquid functionalized on the surface due to liquid-like appearance called supramolecular ionic liquids (SILs).[23, 24]

Graphene as the high specific support has gained more attention in recent years which is due to their extraordinary thermal and mechanical properties, and high stability in different conditions.[25] Three-dimensional graphene-based frameworks (3D-GFs) such as graphene aerogels and foams are important classes of new generation porous carbon materials, which exhibit continuously interconnected macroporous structures, low mass density, high surface area and excellent chemical and physical stability.[26-28] These materials can be served as robust matrices for accommodating metals, metal oxides, and active polymers for various applications. On the other hand, to increase the performance of the support, the chemical modification of the surface with nitrogen doping is a promising method[29, 30].

In this report, combining the superb properties of three-dimensional nitrogen-doped graphene-based frameworks and SILs makes the presented system to be unique for various applications such as DNA delivery. Supramolecular ionic liquid grafted three-dimensional nitrogen-doped graphene (SIL-g-3D-(N)GFs) have several advantages such as high dispersity, functionality, and high surface charge density.[31] Therefore, this system can cause highly dispersive and long term stable graphene-based frameworks in different media.[32, 33]

All chemicals were purchased from Aldrich and used without further purification. IR spectra were recorded on a Bomem MB-Series FT-IR spectrophotometer. Transmission electron microscopy (TEM) analyzes were performed by LEO 912AB electron microscope. Identification and quantification were carried out on a Varian model 3600 gas chromatograph (Varian Iberica, Madrid, Spain) equipped with a split/splitless capillary injection port and flame ionization detector (FID). A CP-Sil-8 fused silica capillary column (25 m_0.32 mm i.d. and 0.52 mm film thickness) from Chrompack was employed. Ultrasonic

bath (EUROSONIC® 4D ultrasound cleaner with a frequency of 50 kHz and an output power of 350 W) was used to disperse materials in solvent. X-ray powder diffraction (XRD) data were collected on an XD-3A diffractmeter using Cu Kα radiation. Hitachi FE-SEM model S-4160 was used for surface imaging. The particle size and zeta potential of the SIL-g-3D-(N)GFs-folate/pDNA complex, were measured using Zetasizer Nano-ZS.

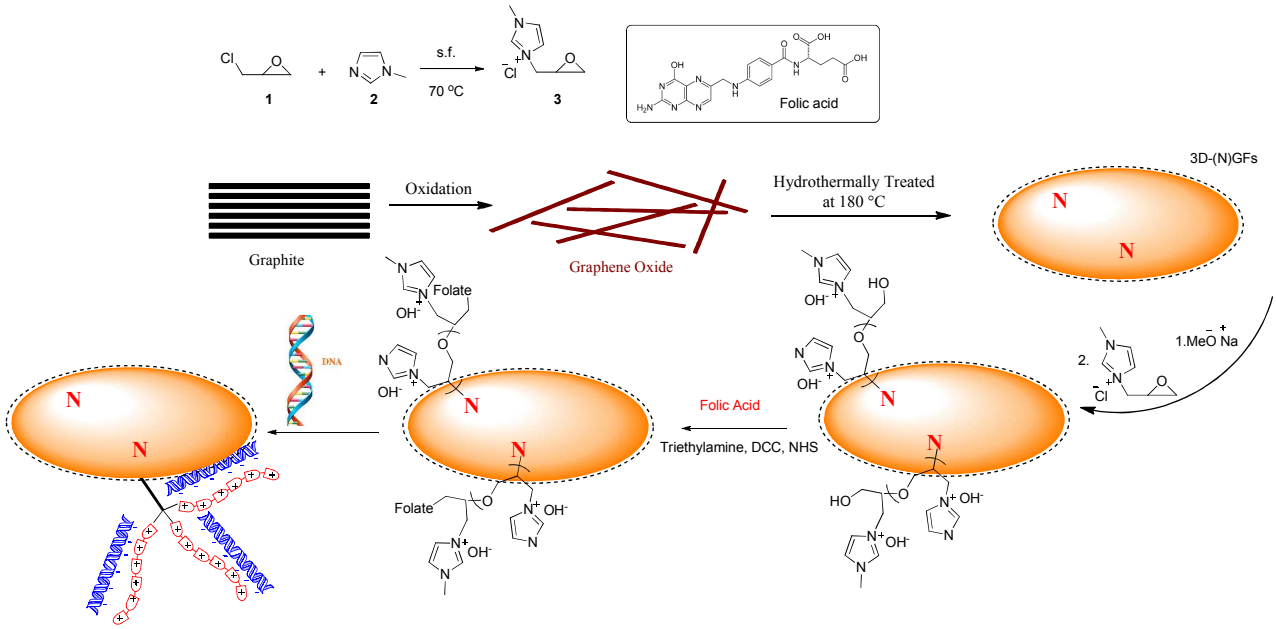
Preparation of 1-methyl-3-(oxiran-2-ylmethyl)-1H-imidazol-3-ium chloride: 2-(Chloromethyl)oxirane (1) (3.9 mL or 50 mmol) and 1-methylimidazole (2) (4.0 mL or 50 mmol) were added to a 100 mL round-bottomed flask equipped with a reflux condenser and reacted for 24–72 h at 70 °C with stirring to the formation of a two-phase mixture under nitrogen atmosphere. The upper layer, which contained mostly the unreacted precursors, was decanted and washed with ethyl acetate for three times and product as a brown liquid was finally obtained.

Preparation of graphene oxide: Graphene oxide (GO) was synthesized by Hummer method.[6] Graphite powder (1.00 g, 325 mesh) and concentrated H_2SO_4 (23 mL) were added to a 250 mL conical flask and the mixture was stirred. Sodium nitrate (0.50 g) was added and the resulting mixture was cooled to 0 °C. Under vigorous agitation, $KMnO_4$ (3.00 g) was added slowly and the mixture was stirred for 1h while the temperature was kept below 35 °C. Then, H_2O (45 mL) was added slowly to the reaction mixture and the solution was stirred for 30 min at 90 °C. Next, H_2O_2 (10 mL of a 30% solution) and deionized water (140 mL) were added to the mixture. Then resulting precipitate was centrifuged and washed repeatedly with HCl (5%, 3 × 15 mL) and EtOH and dried under the vacuum at 60 °C. The GO was obtained as a brown powder.

Synthesis of three-dimensional nitrogen-doped graphene-based frameworks (3D-(N)GFs): 3D-(N)GFs was synthesized with a combined hydrothermal and freeze-drying process.[29] Typically, the as-prepared GO was firstly dispersed in water with ultrasonication reaching a concentration up to 1.5 mg mL^{-1}. A 10 mL aqueous dispersion of GO (1.5 mg mL^{-1}) and 1.2 mmol dicyandiamide was sonicated for 5 min, and then, the mixture was transferred to a Teflon-lined autoclave and hydrothermally treated at 180 °C for 12 h. Finally, the resulting sample was freeze-dried overnight to obtain 3D-(N)GFs.

Synthesis of SIL-g-3D-(N)GFs: 3D-(N)GFs (0.5 g) and cesium hydroxide monohydrate (3.0 g) were placed in a 250 mL Schlenk flask, and 20 mL toluene was added. The mixture was stirred at 60 °C under an argon atmosphere for 1 h to generate the corresponding cesium alkoxide and evacuated at 60 °C for 12 h to remove the toluene and water. Subsequently, 20 mL dry THF was transferred into the Schlenk flask. 1-methyl-3-(oxiran-2-ylmethyl)-1H-imidazol-3-ium chloride was transferred to a graduated ampoule and then transferred into the reaction flask containing the above mixture. The mixture was heated at 60 °C and stirred for at least 12–24 h. The polymer solution was dried under vacuum and dialyzed against deionized water. Finally a highly viscous black liquid like product was obtained. Solid potassium hydroxide was used to replace the chloride ion on the SIL-g-3D-(N)GFs with a hydroxide ion by adding both to water (50 mL) and stirring vigorously at room temperature for 24 h. Then, the mixture was centrifuged, washed with water and dried under reduced pressure to give the supported basic SIL-g-3D-(N)GFs (Scheme 1).

Synthesis of SIL-g-3D-(N)GFs-folate: In a 50 ml flask with a magnetic stirring bar, folic acid (1 g, 2.3 mmol) was dissolved in 30 mL of anhydrous DMSO. Triethylamine (TEA, 0.5 mL), DCC (0.47 g, 2.3 mmol) and NHS (0.26 g, 2.3 mmol) were added subsequently. After 24 h stirring at ambient temperature in the dark, the product was filtered to remove the insoluble byproduct (DCU). A certain volume of FA-NHS solution was added to *SIL-g-3D-(N)GFs* in anhydrous DMSO in the presence of TEA, and the mixture was stirred for 48 h at ambient temperature. The product was dialyzed against DMSO for 4 h, then dialyzed against water for 24 h, which the water was renewed every 6 h, to remove the unreacted folic acid, finally freeze-dried to obtain *SIL-g-3D-(N)GFs-folate.*



Scheme 1 Synthesis of DNA loaded SIL-g-3D-(N)GFs-folate.

Formation of SIL-g-3D-(N)GFs-folate/pDNA complex: The SIL-g-3D-(N)GFs-folate/pDNA complex was performed by the mixing of the SIL-g-3D-(N)GFs-folate aqueous solution with an equal volume of pDNA solution by vortexing for 5 s and incubating for 30 min at room temperature. The complexes were prepared immediately before use.

The synthesis of three-dimensional nitrogen-doped graphene-based frameworks as the chosen support material was accomplished in the following steps as shown schematically in Scheme 1: (i) At first, 1-methyl-3-(oxiran-2-ylmethyl)-1H-imidazol-3-ium chloride was synthesized via the nucleophilic substitution reaction between 2-(Chloromethyl)oxirane and 1-methylimidazole; (ii) GO was synthesized by Hummer method;[34] (iii) The as prepared GO was used to synthesize 3D-(N)GFs by dicyandiamide as the nitrogen precursor *via* a combined hydrothermal and freeze-drying process;[29] (iv) The supramolecular ionic liquid, polyether polyol, on the 3D-(N)GFs was synthesized by a simple nucleophilic reaction between nitrogen groups of three-dimensional nitrogen-doped graphene and 1-methyl-3-(oxiran-2-ylmethyl)-1H-imidazol-3-ium chloride; (v) The exchange of the chloride ions with hydroxide ions was accomplished; (vi) Finally, SIL-g-3D-(N)GFs with cancer cell targeting moieties (folate) was synthesized.

Typical scanning electron microscopy (SEM) image of the suspended GO sheets has been shown in Fig. 1A. SEM is used to obtain information about the morphologies and sample surfaces. The exfoliated graphene sheets with lateral dimensions of several micrometers were observed in SEM images. Fig. 1B shows TEM image of GO, drop casted onto a lacey carbon grid, that it clearly deduced the single layers and very high quality of GO.

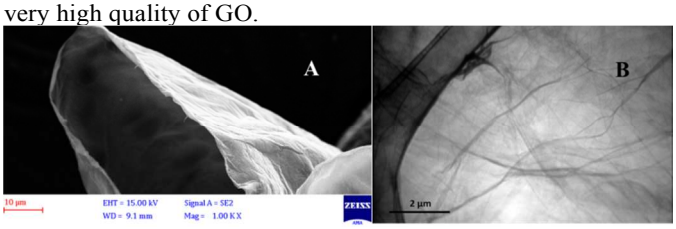


Fig. 1 SEM (A) and TEM (B) images of GO

The 3D morphology and size of as-prepared 3D-(N)GFs were confirmed by SEM images. Fig. 2A and B show an interconnected framework of ultrathin graphene nanosheets with porous structure. As can be seen, the pore size is ranging from a few hundred nanometers to several micrometers. The surface area in porous materials is an important factor affecting the loading of SILs. So, Brunauer-Emmett-Teller (BET) method was used to estimate the surface area of the as-prepared 3D-(N)GFs. Nitrogen adsorption-desorption analysis reveals a typical BET surface area of up to 266.0 m^2 g^{-1} for 3D-(N)GFs. Therefore, the synthesized support has high surface area to embed SILs and subsequently nucleic acid.

The chemical composition of GO, 3D-(N)GFs, and SIL-g-3D-(N)GFs was confirmed by FT-IR, and X-ray powder diffraction (XRD). Moreover, X-ray photoelectron spectroscopy (XPS) was employed for the surface characterization of SIL-g-3D-(N)GFs-folate.

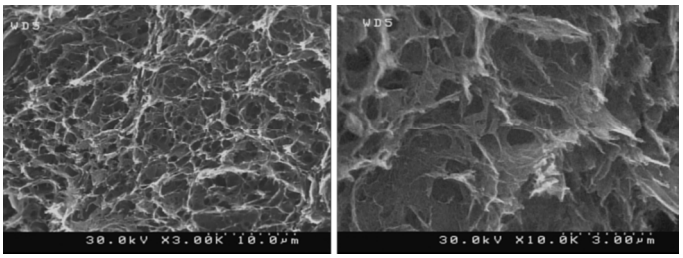


Fig. 2 SEM images of 3D-(N)GFs.

FT-IR spectra of GO, 3D-(N)GFs, and SIL-g-3D-(N)GFs, and SIL-g-3D-(N)GFs-folate after background subtraction are given in Fig. 3A-D, respectively. The spectrum of GO shows bands around 1060 cm^{-1} (ν C-O), 1210 cm^{-1} (ν phenolic), and 1710 cm^{-1} (ν C=O). The results obtained from Fig. 3B shows that nitrogen doping within 3D-GFs has successfully done. Actually, the bands at to 1580, 2040, 2210, and 3470 cm^{-1} are attributed to –C=N and –C–N groups, which are possible in a ring-like structure.[35, 36] After the synthesis of SIL on 3D-(N)GFs, due to the introduction of the imidazolium groups, peaks in the 1430–1640 cm^{-1} region are observed, which confirms the formation of the poly(ionic liquid) on 3D-(N)GFs. FT-IR spectrum of SIL-g-3D-(N)GFs-folate has been shown in Fig. 3D. According to this figure, the bands at 1703, and 3000-3700 cm-1 are respectively related to the (-COO-) and (-NH and -NH2) groups of folate.

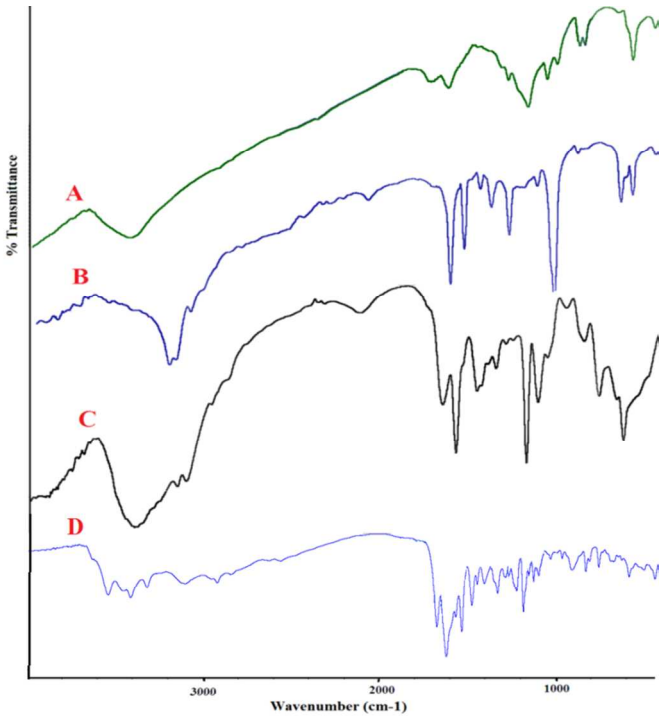


Fig. 3 FT-IR spectra of GO (A), 3D-(N)GFs (B), and SIL-g-3D-(N)GFs (C), and SIL-g-3D-(N)GFs-folate (D).

All samples were characterized by powder X-ray diffraction (Fig. 4). The strong and sharp peak at $2\theta = 10.9^{o}$ corresponds to (0 0 2) of GO representing that graphite is oxidized completely. In the XRD pattern of 3D-(N)GFs, the peak of GO at ~10.9° completely disappeared and the diffraction line at 26.2° was observed suggesting completely and uniformly exfoliation by

the interlayer expansion along the c-axis direction in the 3D-(N)GFs. In the XRD patterns of SIL-g-3D-(N)GFs, and SIL-g-3D-(N)GFs-folate, the characteristic peak of 3D-(N)GFs at 26.2° can be observed which indicated the retained morphology of 3D-(N)GFs after functionalizing with SIL and folic acid.

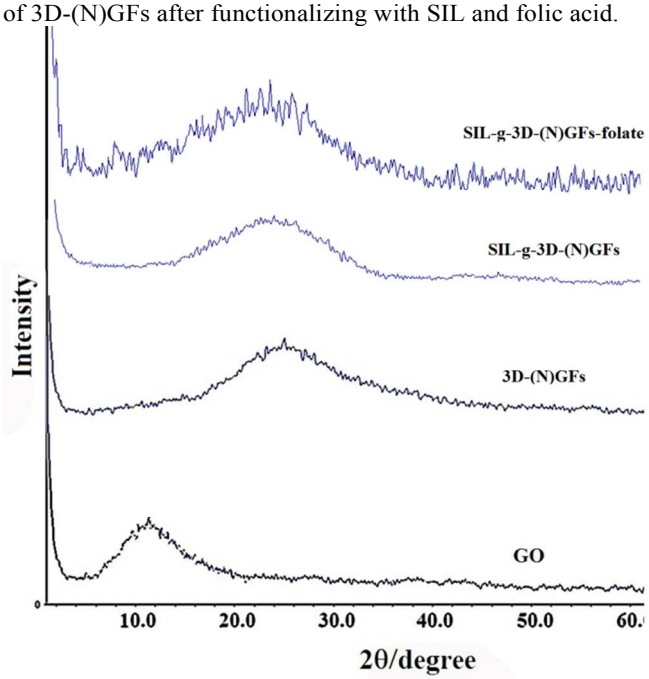


Fig. 4 XRD patterns of GO (A), 3D-(N)GFs (B), SIL-g-3D-(N)GFs (C), and SIL-g-3D-(N)GFs-folate (D).

XPS analysis is often used for the surface characterization of various materials. So, XPS was prepared to further analyze the as-prepared 3D-(N)GFs, and SIL-g-3D-(N)GFs and to examine the composition of their surface. As can be seen in Fig. 5, the peaks C 1s, N 1s, and O 1s present the accurate elemental composition of both materials, which is in good accordance with the designation of the experiment. Moreover, as can be observed, oxygen and nitrogen contents of SIL-g-3D-(N)GFs are more than 3D-(N)GFs, which is clear from their structures.

There are few recent papers on graphene for gene delivery. In 2011, Feng and coworkers successfully used graphene as a non-toxic nano-vehicle for efficient gene transfection. They employed graphene oxide bound with cationic polymer, polyethyleneimine (PEI). According to their results, graphene is a new gene delivery nano-vector with high transfection efficiency and low cytotoxicity.[37] In another work, the linear polyethylenimine-grafted graphene oxide conjugates were prepared and their efficacy to transfer nucleic acids into the mammalian cells was investigated which exhibit transfection efficiency several folds higher than linear and branched PEI with cell viability even higher than linear PEI.[38] In both examples, PEI was used as a cationic polymer to have interaction with DNA. Employing poly(ionic liquid)s as cationic polymers are very novel and unique.

Several experiments have been carried out to show the applicability of the synthesized system for the gene delivery.

The change in surface charge of SIL-g-3D-(N)GFs-folate/pDNA complex was investigated by zeta potential measurements which presented in Fig. 6. At a low mass ratio of 0.1:1, the zeta potential of SIL-g-3D-(N)GFs-folate/pDNA was negative, showing the incomplete complexation. With higher mass ratio, the zeta potential increased rapidly and reached a plateau at about 52 mV.

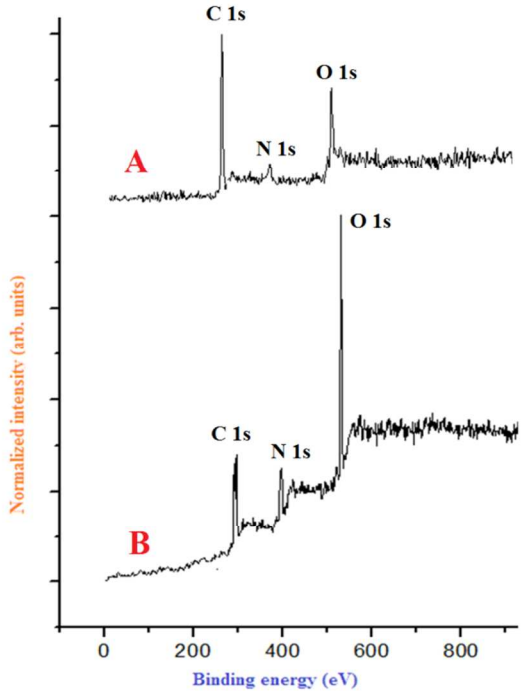


Fig. 5 XPS spectra of 3D-(N)GFs (A), and SIL-g-3D-(N)GFs (B).

The positive surface charge of SIL-g-3D-(N)GFs-folate/pDNA complex can facilitate the attachment of the complex to the negatively-charged cellular membranes and subsequently assists the cellular uptake of pDNA complexes.[39]

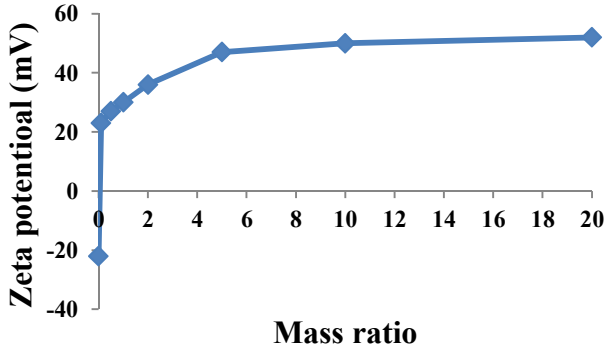


Fig. 6. Zeta potential of SIL-g-3D-(N)GFs-folate/pDNA complexes at different mass ratio: 0.1 : 1, 0.5 : 1, 1 : 1, 2 : 1, 5 : 1, 10 : 1, and 20 : 1, respectively.

The size of the SIL-g-3D-(N)GFs-folate/pDNA complex at various mass ratios was measured using dynamic light scattering (Fig. 7). The average size of the SIL-g-3D-(N)GFs-folate/pDNA complexes at mass ratios ranging from 1.2 : 1 to 20 : 1 varied between 310 and 490 nm. So, as the complexes with positive charge and small size are favorable for cellular uptake and intracellular trafficking, the SIL-g-3D-(N)GFs-folate could be a suitable carrier in DNA delivery. In general, cells uptake exogenous materials like nanoparticles through endocytosis.[40, 41]

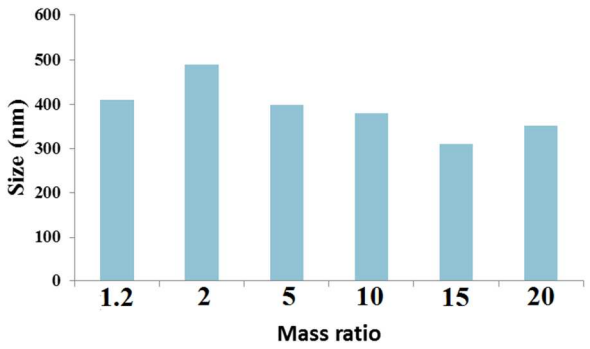


Fig. 7. Average sizes of SIL-g-3D-(N)GFs-folate/pDNA complexes at different mass ratios: 1.2 : 1, 2 : 1, 5 : 1, 10 : 1, 15 : 1 and 20 : 1, respectively.

Conclusion

The approach of this research is the synthesis and introducing a super dispersible cationic polymer. This system contained three-dimensional nitrogen-doped graphene-based frameworks as the support and supramolecular imidazolium-based ionic liquid as the cationic polymer. Moreover, the as-prepared material was functionalized with folates which are cancer cell targeting moieties. The mentioned advantages of this system make it promising candidate to be employed in the biological purposes such as DNA delivery. Further investigations of the application of this system are currently in progress in our lab.

Notes and references

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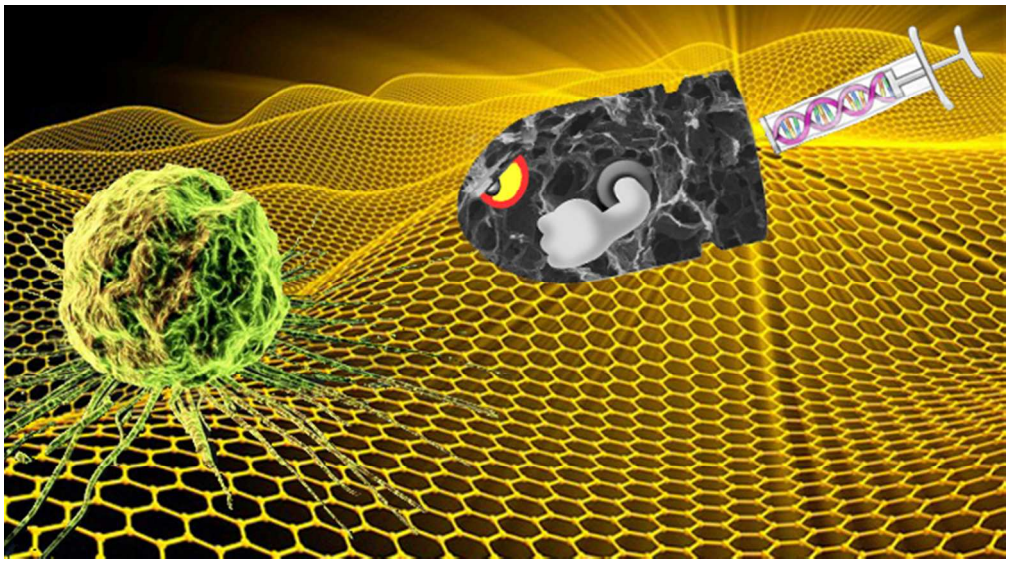

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