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# Pluronic – Grafted Copolymers as Nanoplatforms for Effectively Delivering Hydrophobic Anticancer Drugs

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***Authors' contributions***

*This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Several kinds of anticancer drugs have significantly contributed in cancer therapy but these drugs exhibited several side-effect. There has recently been an emerging approach in drug development by which efficient exploitation of using nanocarriers. The drug delivery nanocarriers are considering as a sustainable and innovative development. In these studies, two kinds of pluronic-conjugated polymers were prepared in a green synthetic process via conjugate of thermosensitive copolymer pluronic F127 (F127) derivative onto amine-functionalized generation 4.0 polyamidoamine (PAMAM G4.0) dendrimer or heparin. The pluronic–functionalized polymers (G4.0-F127 and Hep-F127) were

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characterized by Fourier Transform Infrared Spectroscopy (FT-IR), Gel permeation chromatography (GPC), and Transmission Electron Microscopy (TEM), which revealed that size of these nanocarriers were below 180 nm in diameter. The G4.0-F127 nanocarriers exhibited a high entrapment-efficiency (EE) of 5-fluorouracil (5-FU), approximately 71.35±1.75% of fed drug, which was significantly higher than that of PAMAM G4.0 at 42.18±1.89% and F127 at 18.75±2.25%. For Hep-F127, the nanocarriers exhibited a high drug loading efficiency with 5-FU, Erlotinib hydrochloride (Erlo) and Cisplatin (Cis) at 37°C. Releasing studies indicated that the nanocarriers could be used for delivering several kinds of hydrophobic drugs and the drug-loaded systems have showed a significantly antiproliferative activity. The obtained results demonstrated that F127-conjugated polymers could be potential nanocarriers for drug delivery systems.

*Keywords: Dendrimer; heparin; pluronic; nanocarrier; anticancer drug; green synthetic process.*

## 1. INTRODUCTION

In oncology research, development research for new drugsand efficient exploitation of the drugs in the delivery systems have been leading the development trend. Drug delivery systems have been considering as a sustainable and innovative development in medical applications in which drugs can be targeted into tumors, resulting in reducing side-effect that could contribute to rise the quality of human life. Among the delivery systems, polymeric nanocarriers like nanogels and dendrimers have been expected as a promising platform for various therapeutics agents. The nanocariers exhibited a significant enhancement of water solubility, drugs-storage stability, anti-tumor activity and reduction of side-effects of anticancer drugs [1-6]. PAMAM-based nanocarrier contains internal cavities, which could be utilized for delivering several bioactive molecules. Moreover, the dendrimer also contains functional groups which could be grafted with targeting factors [7,8]. Theaminated PAMAM dendrimers also reported with high cytotoxicity due to its positive charge [9-11]. Pegylation or alkylation of the dendrimers was utilized to improve biocompatibility or solubility of the hydrophobic or cationic drugs [12-15]. Besides the development of dendrimer-based nanocarriers, polysaccharide-based nanogels also were potentially effective candidates such as chitosan and heparin nanogel [16-19]. Incorporation of these polysaccharide in nanogels could enhance its biocompatibility and biodegradation as well as delivery of charged drugs. For enhancing drug loading, several kinds of thermosensitive amphiphilic (co)polymers like poly (N-isopropylacrylamide) (p-NiPAM), polylactide-co-glycolide (PLGA), pluronics were ultilized to conjugate on these dendrimers and polysacharides which exhibited a great drug loading efficiency and sustainable releasing. It was reported that the amphiphilic polymers could be well-loaded several kinds of

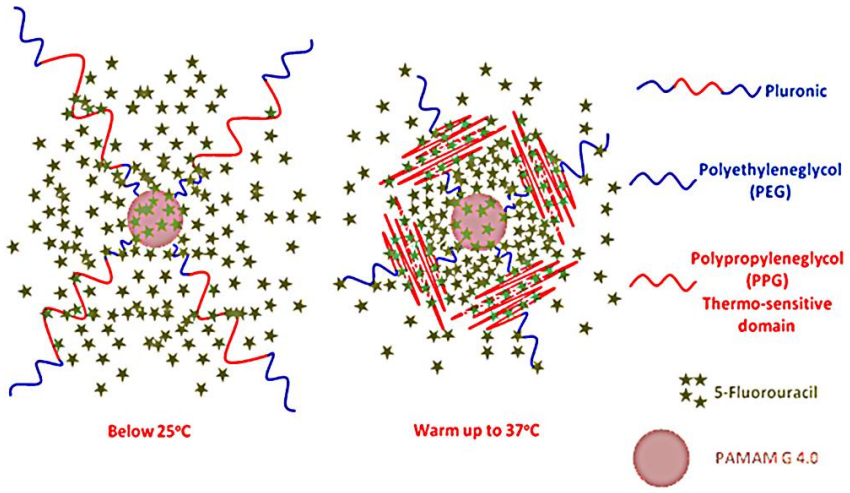


**Fig. 1. Nanogel formation mechanism encapsulated 5 FU anticancer drug in the hydrophobic domain and inner cavities**

hydrophobic drugs. In fact, Stevanovic et al. [20] introduced several kinds of PLGA-based nanoparticles into effectively delivering rifampicin, vincristine sulfate and paclitaxel. Also, p-NiPAMshowed a high curcumin loading capacity, reaching to 86% of total fed drug and a prominent controlled release system [21]. Pluronic-based nanocarrier encapsulating doxorubicin has been highly expected to clinical trial [22]. Simultaneously, the conjugation of thermosensitive polymers into several biological polymers has been an emerging approachon developing the novel grafted copolymers that could effectively deliver anticancer drugs or proteins [23,24].

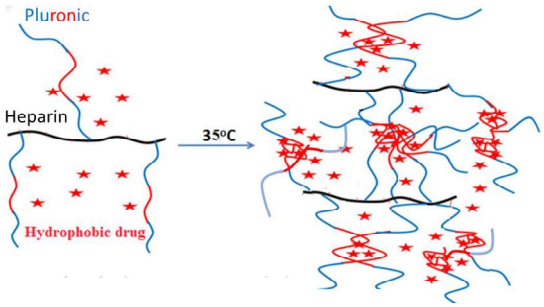


**Fig. 2. Preparation of 5-FU loaded Hep-F127 nanogel**

These studies introduced pluronic-based nanocarriers (G4.0-F127 and Hep-F127) at which utilized hydrophobic interaction of pluronic chains with hydrophobic drugs and internal cavity of these nanogels to increase drug loading capacity and control its delivery system (as demonstrated in Fig. 1 and Fig. 2). The grafted copolymer was characterized its structure then evaluated the morphology, drug loading-releasing capacity as well as inhibition capability against human breast cancer MCF-7 cell line. The drug nanocarriers would be expected to reduce side-effects of free anticancer drugs due to their prominently controllable release.

## 2. MATERIALS AND METHODS

Pluronic F127 (F127), 5-fluorouracil (5-FU), and Cisplatin (Cis) (300.05 MW, 99.999%) were purchased from Sigma. Erlotinib hydrochloride (Erlo) was ordered from LC Laboratories. 4-nitrophenyl chloroformate (NPC), Tyramine (TA), Heparin sodium (Low molecular weight), Aminopropanol, 1,4-diaminobutane, Tetrahydrofuran (THF), 1-ethyl-3-3-dimethylaminopropyl carbodimide (EDC), N-hydroxysuccinimide (NHS) were obtained from Acros Organics (Belgium). PAMAM G4.0 was prepared following the procedure reported by Tomalia [7,13]. Regenerated cellulose dialysis bags (MWCO 3,500-5,000 Da and 12,000-14,000 Da) were bought from Spectrum Laboratories Inc.

### 2.1 Synthesis of G4.0-F127

PAMAM derivative was prepared through three periods as demonstrated in Fig. 3. First, F127 was activated in a free solvent process. Brieftly, the copolymer (6.30 g; 0.50 mmol) was melted under vacuum at 75°C, then was activated by NPC (0.30 g; 1.50 mmol) under stirring for 4 hours. The reaction was adjusted into room temperature and diluted by 15 mL of ethanol. The polymer solution was dialyzed in ethanol to obtain NPC-activated pluronic F127. $^1$H NMR result indicated typical peaks at δ ppm= 1.15 (-$CH_3$/F127), 3.64 (–$CH_2$–$CH_2$–, F127), 4.40 (–$CH_2$–O–NPC, F127), 7.39 - 8.27 ($H_{arom}$, NPC). Degree of activation was over 97% by Proton Nuclear Magnetic Resonance ($^1$H NMR) (Fig. 4).

To substitute one of two NPC groups of NPC activated F127, TA (0.07 g; 0.51mmol) was dissolved completely in ethanol, and added drop-wise into 30 ml of ethanol solution containing the NPC-activated F127 (5.43 g, 0.42 mmol) and then was stirred overnight. The reaction mixture was concentrated and dialyzed in ethanol to obtain NPC-activated F127, filtered, and dried in vacuum condition to obtain a NPC-F127-TA polymer. TA substituted about 54% of NPC in the activated F127. $^1$H NMR ($CDCl_3$) of NPC-F127-TA, δ ppm = 1.15 (-$CH_3$/F127), 3.64 (–$CH_2$–$CH_2$, F127), 6.90 - 7.10 ($H_{arom}$, TA), 7.39 - 8.28 ($H_{arom}$, NPC) (Fig. 5).

To obtain the pluronic-functionalized dendrimer, 20 ml of ethanol solution of NPC-F127-TA (2.92 g; 0.23 mmol) was added drop-wise into the PAMAM G4.0 (0.20 g; 14 µmol in methanol) under stirring for 24 hours. After this time, the mixture was dialyzed against methanol in 3 days to obtain a G4.0-F127 copolymer. $^1$H NMR ($CDCl_3$) of G4.0-F127-TA, δppm =2.60- 2.80 (-$CH_2CH_2CO$-, PAMAM G4.0), 3.30-3.60 (O-$CH_2$-$CH_2$-O, O-$CH_2$-CH-O, pluronic), 3.70(-$CONHCH_2CH_2N$-, PAMAM G4.0), 6.90 and 7.10 (–CH=CH–, TA) (Fig. 6).

### 2.2 Preparation of Hep-F127 Copolymer

Hep-F127 copolymer was synthesized via three steps. Firstly, heparin solution (400 mg in distilling water) was activated with EDC (0.66

mmol) and NHS (200 mg) and then partially aminated by 1,4-butanediamine (1.34 mmol) for 24 hrs at 35°C. The mixture was dialyzed against distilling water for 3 days and lyophilized to obtain an aminated heparin (402 mg).

Secondly, mono NPC-activated F127-OH was prepared to further conjugate with the aminated heparin producing Hep-F127. Amino propanol (7.32 mmol in 20 ml THF) was slowly added into NPC-F127-NPC solution (8.13 mmol in 30 ml THF) under stirring for 24 hours. The mixture was precipitated in diethyl ether solvent to obtain mono NPC-activated F127-OH. Finally, the NPC-activated F127-OH (25 µmol) was conjugated to the aminated heparin (350 mg) under stirring for 24 hrs. The obtained solution was dialyzed against DI water for 2 days and lyophilized to obtain Hep-F127. $^{1}$H NMR ($D_2O$) of Hep-F127 δppm, 2.8 ppm- 3.15 ppm (protons of Hep), 1.08 ppm (protons of PPO block) and 3.62 ppm methine proton (Fig. 7).

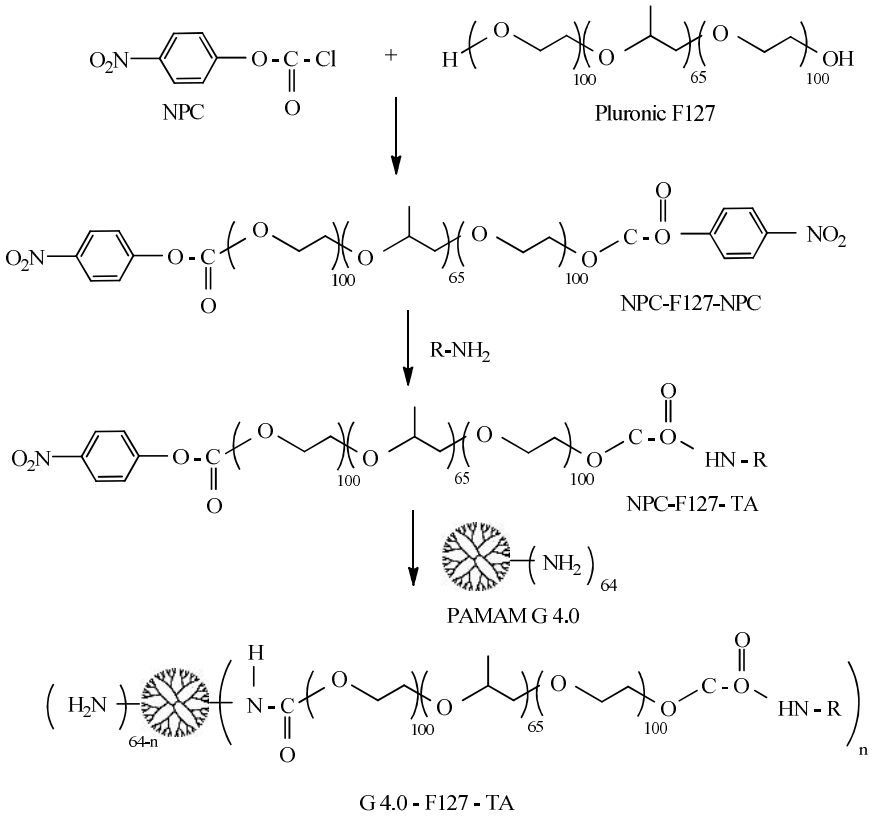


**Fig. 3. Synthetic scheme of G4.0-F127**

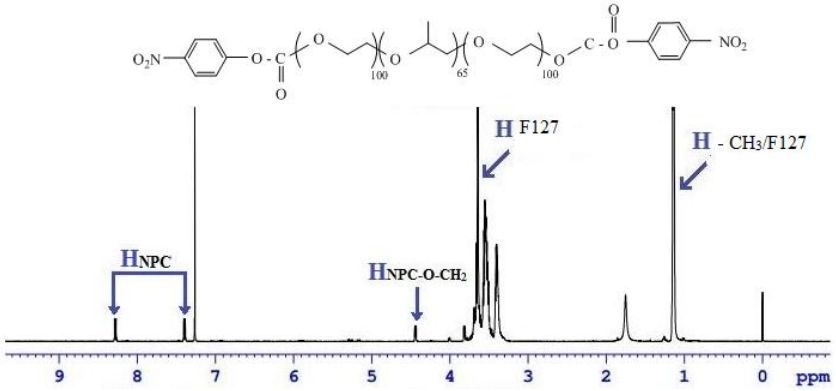


**Fig. 4. $^{1}$H-NMR spectra of NPC-F127-NPC**

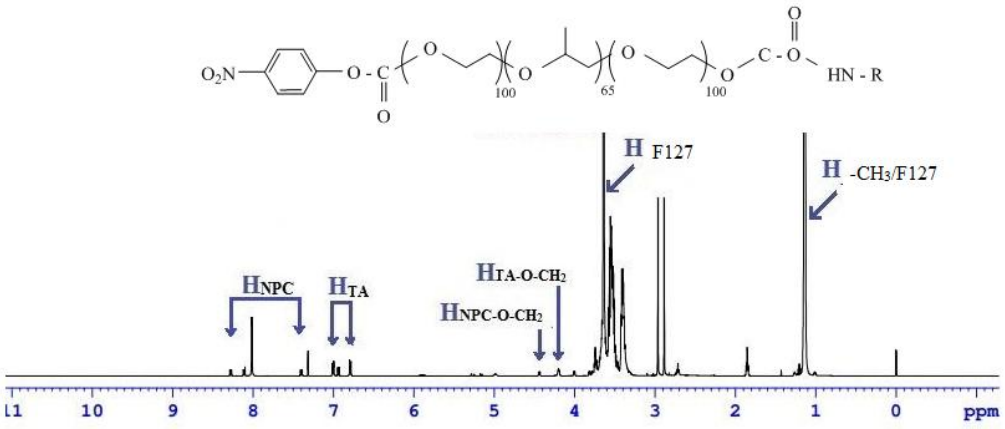


**Fig. 5. [1]H-NMR spectra of NPC-F127-TA**

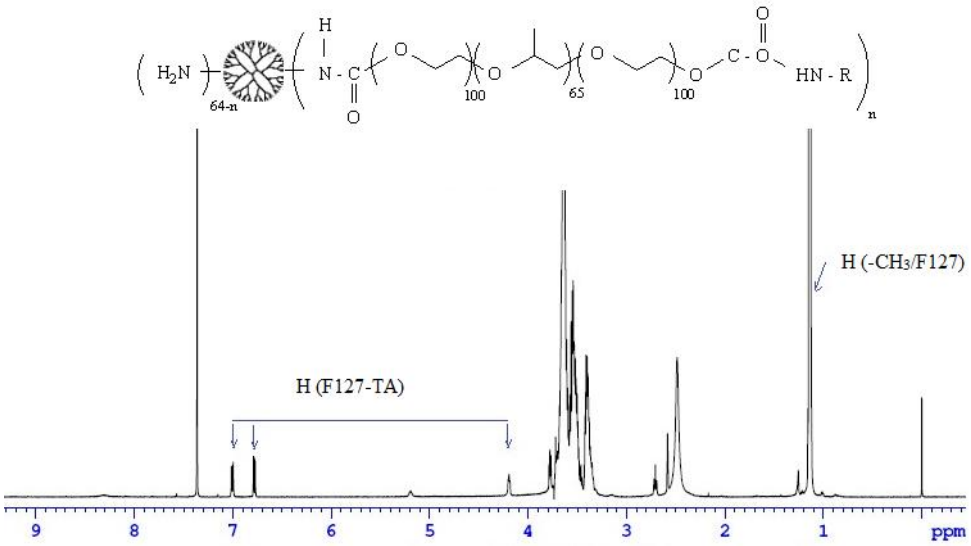


**Fig. 6. [1]H-NMR spectra of G 4.0 – F127 – TA**

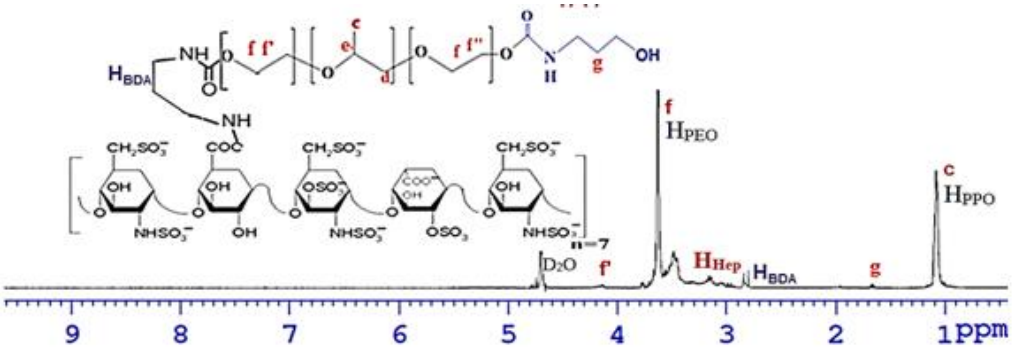


**Fig. 7. [1]H-NMR spectra of Hep-F127 copolymer**

### 2.3 Preparation of the 5-FU-loaded Nanocarriers

5-FU drug (80 mg) was dissolvedin pluronic-grafted copolymer solutions (400 mg in distilling water) under stirring at room 25°C for 24 hours. The mixtures were incubated in 37°C and then dialyzed against distilling water three times to remove unloaded 5-FU. The dialyzed solutions were freeze-dried to achieve 5FU-loaded pluronic copolymers. Amount of loaded 5-FU drug entrapment and loading efficiencies was determined by High-performance Liquid Chromatography (HPLC) measurement regarding the fed 5-FU and the unloaded 5-FU in totally dialyzed solutions [25,26]. Morphologies of the nanocarriers and drugs-loaded nanocarriers were observed by TEM.

### 2.4 *In vitro* Release Kinetics

The drugs loaded G4.0-F127 or Hep-F127 nanocarrier were dispersed in PBS (pH 7.4) and then added into membrance dialysis bags (3500 MWCO) to evaluated release kinetics at both condition pH (7.4) and pH (5.5) following dynamic dialysis technique. Released amount of 5-FU or Erlo was defined at time intervals by HPLC and release of Cis was measured by ICP-AES instrument.

### 2.5 Cytotoxicity Assays

Cytotoxicity tests were conducted Sulforhodamine B colorimetric assay. PAMAM G4.0, G4.0-F127 and 5-FU loaded G4.0-F127 were evaluated with antiproliferative activity of MCF-7 breast cancer cell. For Hep-F127 nanogels, the nanocarrier was loaded Cis, Erlo and 5-FU in turn, and then evaluated antiproliferative activity with human lung cancer cell line NCI-H460. The tested samples were cultured in a humidified 5% $CO_2$ incubator at the screening concentration of 100 μgml $^{-1}$ and different concentrations of the loaded drugs to confirm inhibitory concentration 50% of cell growth ($IC_{50}$).

## 3. RESULTS AND DISCUSSION

### 3.1 Characterizations of G4.0-F127 and Hep-F127

Characterization of G4.0-F127: $^1$H NMR results indicated that hydroxyl groups were well-activated and NPC-pluronic-TA was also conjugated to PAMAM G4.0. The formation of the G4.0-F127 was determined by FT-IR spectroscopy. Characteristic absorption band for vibration frequency of $NH_2$ and NH are at 3423 cm$^{-1}$, amide groups (HNC=O) give absorption bands at 1646 cm$^{-1}$. Strong C-O-C stretching absorption at 1112 cm$^{-1}$ (C-O-C) (Fig. 8). Molecular weight of the G4-F127 was determined in comparison with pluronic-F127 (Fig. 9). Molecular weight of the G4-F127 is around 353,300 Da, this value is significantly differ from molecular weight of F127 (Mw: 12,600 Da). This meant that the PAMAM G4.0 was decorated by 27 pluronic molecules, approximately.These results indicated the efficiency of the synthetic process of G4.0-F127 copolymer for drug delivery system.

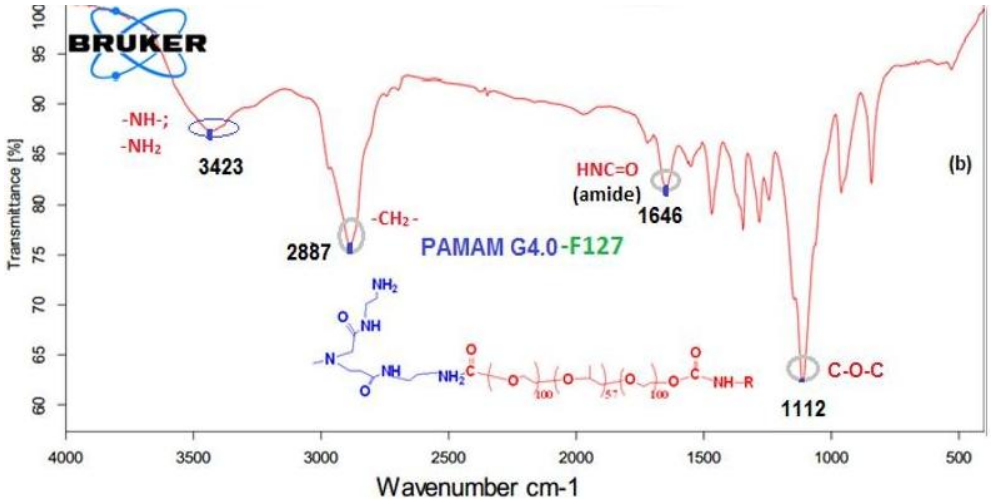


**Fig. 8. FT-IR spectrum of G 4.0 – F127**

The F127 and G4.0-F127 copolymer could be well-observed around 15 nm (Fig. 10.a) and in the range of 120 to 180 nm (Fig. 10b), respectively. In comparison with the theoretical size of F127 and G4.0-F127, there was a significant size increment in the G4.0-F127 which could confirm a success in preparation of the thermosensitive nanocarrier.

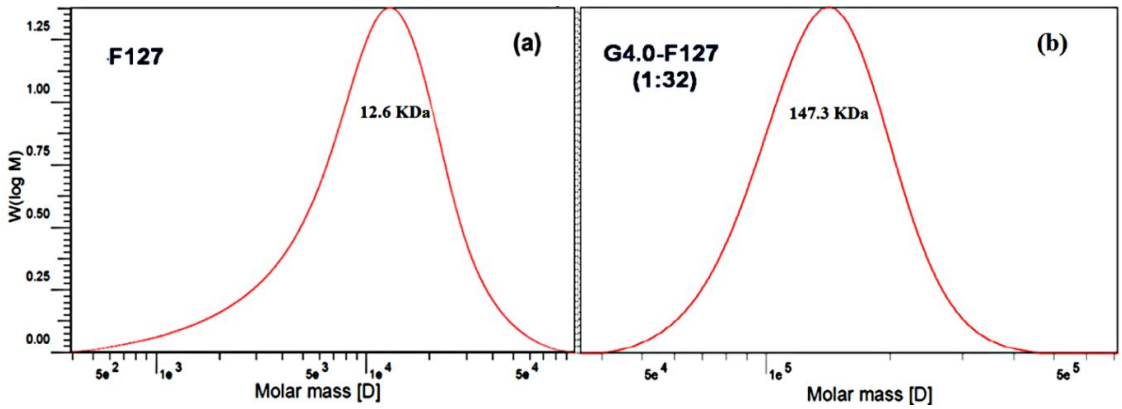


**Fig. 9. GPC results of pluronic F127 (a) and G4.0-F127 (b)**

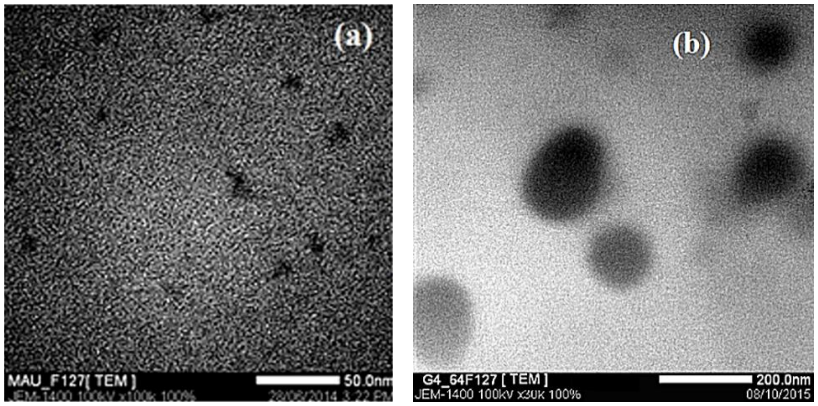


**Fig. 10. TEM images of pluronic F127 (a), G4.0-F127 (b)**

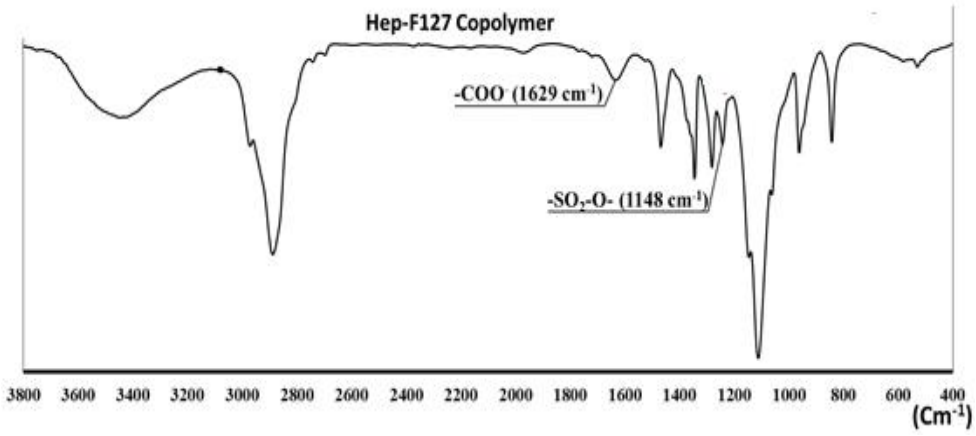


**Fig. 11. FT-IR spectrum of Hep-F127**

Characterization of Hep-F127: According to typical signals from $^{1}H$ NMR spectrum, it could confirm that Hep-F127 obtained via conjugation of mono NPC activated F127 onto the aminated. Moreover, FT-IR spectrum, 1629 $cm^{-1}$(-COO- scissor) and 1148 $cm^{-1}$ ($SO_2$-O- scissor) of the F127-grafted copolymer were recorded (Fig. 11). The copolymer could aggregate forming nanogelswith size distribution at 114 nm by DLS when the solution was heated to 35°C (Fig. 12). The size distribution confirmed efficiency in the modification of copolymer.

### 3.2 Drug Encapsulation in Nanocarriers

Obtained results from drug-encapsulated G4.0-F127 indicated the higher drug loading efficiency (reaching to 71.35±1.95% of the fed drug) in comparison with, PAMAM G4.0 (42.18±1.89%) and F127 (18.75±2.25%). The highest amount of drug loading in G4.0-F127 could be explained that the drug and the hydrophobic domain of F127 interact each other resulting in enhancing drug entrapment in the inner G4.0-F127 nanogels.

For Hep-F127, carboxylated groups of the copolymer could complex with the hydrated Cis and then aggregated forming nanogels.The Hep-F127 nanogel indicated a higher drug-carrying capacity of the Cis (over 42 wt/wt%) in comparison to other previous study on carboxylated PAMAM dendrimer carrying the aquated Cis (below 28wt/wt%) [8-10]. The Cis-loaded Hep-F127 nanogels exhibited size ranging from 80 to 100 nm by TEM and its size distribution at 134 nm by DLS as shown in Fig. 13. The Hep-F127 nanogelsalso exhibited high loading efficiency of 5-FU (80.24 wt/wt%) and Erlo (26.12 wt/wt%). These results perform potential of the pluronic-based nanogels in loading several kinds of hydrophobic drugs.

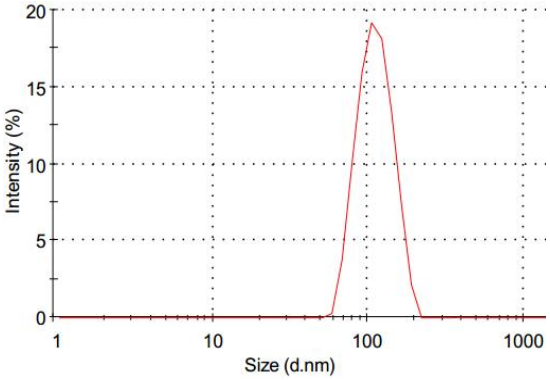


**Fig. 12. Size distribution of the aggregated Hep-F127 nanogel**

### 3.3 Drugs Release Behavior from the Nanocarriers

In the study, we evaluated three of hydrophobic drugs releasing from F127-decorated nanocarriers in two physiological and tumor conditions. In Fig. 14, 5-FU encapsulated G4.0-F127 shows a sustainably released behavior of the drug after 96 hours while the drug is fast release in control sample that is approximately 100% of the released 5-FU in the initial stage.

Fig. 15 indicates that three kinds of anticancer drugs slowly released from the drug-loaded Hep-F127 nanocarriers after 72 hours. However, there is different in releasing behavior of these drugs. Cis exhibited a higher released amount upto 54% in comparison with 5-FU and Erlo at the same time. The phenomenon could be

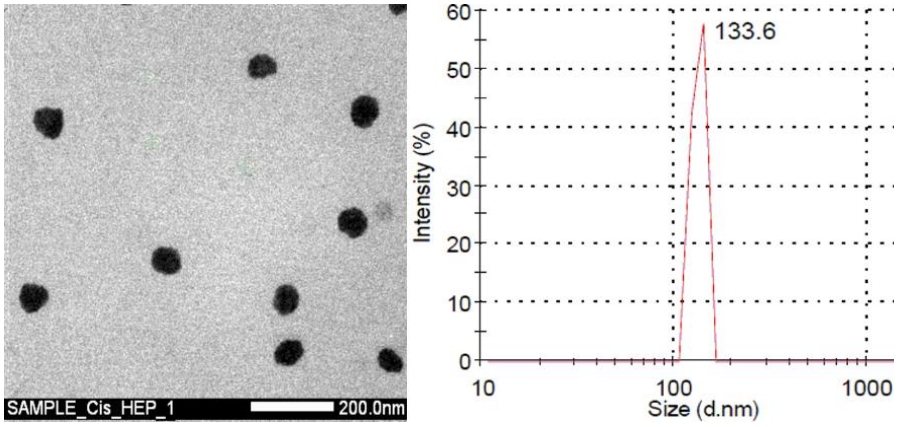


**Fig. 13. Morphology of Cis-loaded Hep-F127 by TEM and its size distribution**

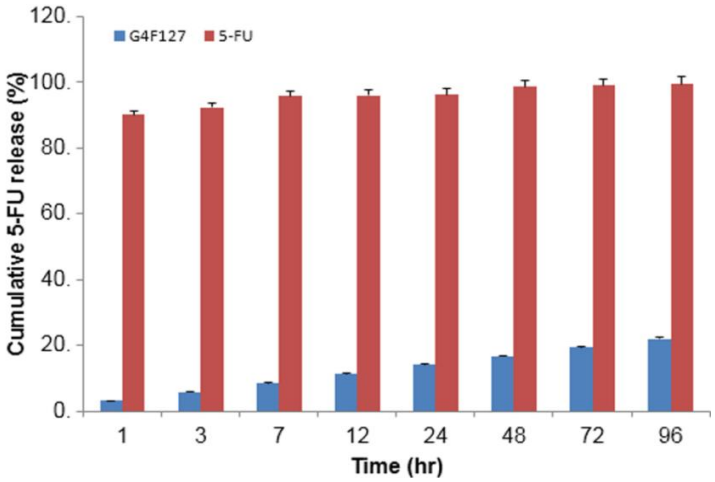


**Fig. 14. Release profile of 5-FU from G4.0-F127**

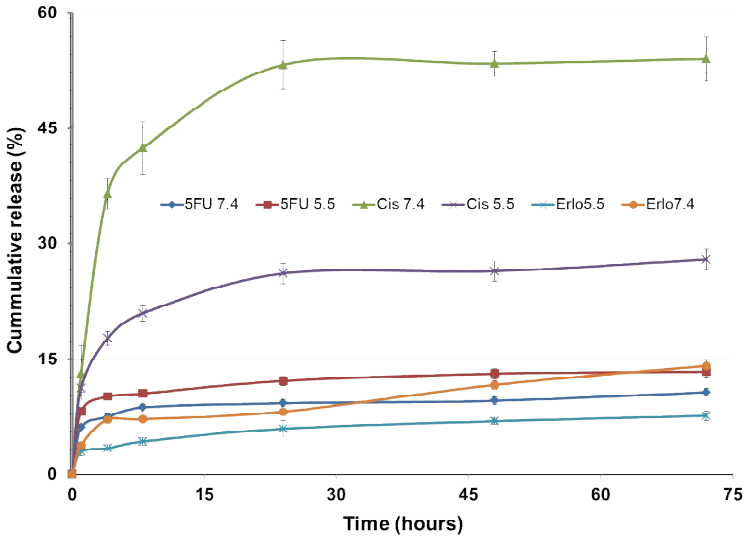


**Fig. 15. Release profile of 5-FU, Cis and Erlo from Hep-F127 nanogel**

explained that the complexation of the hydrated Cis and Hep-F127 is low stability. Moreover, the hydrated Cis is more hydrophilic than that of 5-FU and Erlo resulting in reducing encapsulation of the drug in the nanogel.

In general, release profiles of three investigated drugs are sustainable from two pluronic-decorated nanocarriers. This is significant to exploit these nanogels for delivering several kinds of hydrophobic drugs.

## 3.4 Cytotoxicity Assays

*In vitro* studies indicated that PAMAM G4.0 was cytotoxic at screening concentration (100 $\mu gml^{-1}$) which inhibited 35.49±3.93% of MCF-7 cell growth. While G4.0-F127 (100 $\mu gml^{-1}$) inhibited 4.20±0.45% of cell growth that meant nanocarriers were nontoxic. The modification can reduce the cytotoxicity of PAMAM 4.0. The result also shown that 5-FU had a highly antiproliferative activity with $IC_{50}$ values at 1.63±0.01 µg/ml approximately. However, 5-FU-loaded G4.0-F127 could dramatically reduce the cytotoxicity of the drug ($IC_{50}$ values at 3.98±0.02 µg/ml) as shown in Table 1.

Cytotoxicity of Hep-F127 and antiproliferative activity of three drugs (Cis, Erlo and 5-FU) against NCI-H460. The study showed that Hep-F127nanogels was nontoxic with the cell (Table 2). The table also shows that these anticancer drugs are highly toxic. IC50 value of

**Table 1. Cytocompatibility of G4.0-F127 and antiproliferative activity of the drug-loaded nanocarrier**

| Sample | Conc. (µg/ ml) | Antiproliferative activity (% MCF-7 cell growth) |
|---|---|---|
| PAMAM G4.0 | 100 | Inhibited 35.49 ± 3.93 |
| G4.0-F127 | 100 | Inhibited 4.2 ± 0.45 |
| 5-FU | 1.63 | Inhibited 50 |
| 5-FU-loaded G4.0-F127 | 3.98 | Inhibited 50 |

**Table 2. Cytocompatibility of Hep-F127 and antiproliferative activity of the drugs-loaded nanocarriers**

| Sample | Conc. (µg/ ml) | Antiproliferative activity (% NCI-H460 cell growth) |
|---|---|---|
| Hep-F127 | 100 | Inhibited 2.98 ± .139 |
| Cis | 0.51 | Inhibited 50 |
| 5-FU | 1.11 | Inhibited 50 |
| Erlo | 0.85 | Inhibited 50 |
| Cis-loaded Hep-F127 | 5.70 | |
| 5-FU+G4.0-F127 | 2.77 | Inhibited 50 |
| 5-FU+G4.0-F127 | 5.80 | Inhibited 50 |

Cis, 5-FU and Erlo respectively obtained 0.51, 1.11 and 0.85 µg/ml. However, the drugs-loaded nanogels significantly decreased cytotoxicity of the drugs with $IC_{50}$ at 5.70 µg/ml for Cis-loaded nanogel, 2.77 µg/ml for 5-FU-loaded system and 5.80 for erlo-loaded Hep-F127 nanocarrier.

These results partially performed efficancy of pluronic-decorated nanocarriers in reducing cytotoxicity of these investigated anticancer drugs.

## 4. CONCLUSIONS

This study presented a green synthetic process to successfully prepare G4.0-F127 and Hep-F127 nanocarriers for drug delivery. The positive results demonstrated that G4.0-F127 and Hep-F127 could be two potential nanocarriers for delivering several kinds of hydrophobic drugs and would make a promising foundation for delivering dual anti-cancer drugs in the future. Furthermore, these results could offer potentials of the nanocarriers for drugs delivery *in vivo* ontumor-xenografted animal models.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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