pH-Sensitive Nanoaggregates for Site-Specific Drug-Delivery as Well as Cancer Cell Imaging

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ABSTRACT: Multifunctional polymeric nanoaggregates could enable targeted cancer therapy and imaging, which eventually facilitate monitoring of the therapeutic effect. A fluorescent nanoaggregate is constructed for theranostic application. Chlorambucil (Chl), a fluorescent inactive chemotherapeutic agent, is covalently attached to the nanoaggregate for therapeutic action. The pyrene (Py) motif is also covalently attached to the nanoaggregates, with the motivation of cancer cell imaging. This nanoaggregate is further functionalized with biotin (Bt) for receptor-mediated drug delivery. The efficiency of this system is evaluated by in vitro cell studies to prove its receptor-mediated internalization as well as theranostic capabilities. This newly designed nanocarrier, Nor−Chl−Py−Bt (Nor, norbornene), has the ability to combine both therapeutic and diagnostic capabilities into a single polymer that offers existing prospects for the development of nanomedicine.

INTRODUCTION

Theranostics is a concept that deals with the integration of therapy and diagnostics.1–3 Cancer treatment gets delayed due to the absence of proper tools for the early stage detection, which reduces survival rates.4 Among the various cancer treatments currently practiced, chemotherapy continues to be the best option, primarily because of its high efficiency.5 There are a number of anticancer drugs available, among which chlorambucil (Chl) is an efficient chemotherapeutic agent against various cancers.6–9 Chl belongs to a class of DNAalkylating agents that has been proven to clinically important for the treatment against various cancers.6–9 Chl exhibits its mode of action through the alkylation of the N7 atoms of guanine or adenine as well as the N3 of adenine in double-helical DNA, which leads to apoptotic cell death through the accumulation of persistent DNA damage.6–9 However, despite its several advantages, Chl suffers from several limitations, including drug solubility, stability, selectivity, and resistance.5 Adverse side effects on healthy tissues, multidrug resistance, and several other factors have restricted the use of Chl in conventional chemotherapy.

There are a number of prodrug systems to overcome this obstacle, for example, nanoparticles, liposomes, and polymers, among which polymeric systems are always preferable due to their advantage of including many functional groups, which can overcome the poor pharmacokinetics, inappropriate distribution, and poor solubility and facilitate faster clearance of the drugs.5,10 The ability to include a receptor moiety for site specificity makes the system a highly potential candidate in anticancer therapeutics, as this moiety can recognize cancer cells from among healthy cells, which enables systematic distribution of the drug inside the body.5 Chl is nonfluorescent in nature because of which it is difficult to monitor its therapeutic path in the cell in vitro study.4–20 To overcome this, we have designed a prodrug system that contains the drug along with pyrene (Py), which helps monitor the system under a fluorescence microscope during in vitro studies.7,11–15,16 For site-specificity and longer blood circulation, we have introduced biotin (Bt)-derived polyethylene glycol (PEG), as cancer cell lines show overexpression of the Bt receptor.16–18,22

RESULTS AND DISCUSSION

Toward the goal of site-specific therapy as well as tracking of the nonfluorescent chemotherapeutic drug Chl, we designed three different monomers, namely, Mono 1, Mono 2, and Mono 3 (Scheme 1). The formation of Mono 1, Mono 2, and Mono 3 was completely characterized by 1H NMR and 13C NMR spectroscopy (Figures 1 and S1–S10). The two-step synthesis of Mono 1 was characterized using 1H NMR and 13C NMR spectroscopy (Figures 1 and S1–S3). In the case of I, the alcoholic proton (−OH) appeared at δ = 4.7 ppm in DMSO-d6 (Figure S1). The formation of Mono 1 was confirmed by the disappearance of the signal due to −OH at 4.7 ppm, and all other new peaks appeared corresponding to Chl, which clearly confirmed the formation of Mono 1 (Figure 1a). The 13C NMR spectrum also clearly proved the formation of the product, as a new signal arose at δ = 172 ppm (Figure S3). The formation of Mono 1 was also confirmed by the

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Scheme 1. Synthesis of Mono’s 1–3
electrospray ionization mass spectrometry (ESIMS) technique (Figure S16). The synthesis of Mono 2 was also a two-step process, starting from commercially available exo-5 norbornene carboxylic anhydride. The anhydride was reacted with ethanol amine to get 1 as a pure white crystalline product (Figures S1 and S2). This product upon reaction with 4-(1-pyrenyl) butyric acid in the presence of the reagents N,N’-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) gave norbornene (Nor)–Py (Mono 2). The formation of Mono 2 was confirmed by NMR spectroscopy and the ESIMS technique (Figures 1b, S4, and S17). For making the system water soluble, we synthesized Nor–PEG–Btn (Mono 3). To attach the Btn moiety, we first prepared amine-terminated Nor–PEG. For that, we synthesized boc-protected glycine (2) (Scheme 1). Boc anhydride was used to block the amine group of the glycine (1.05 equiv), which further reacted with PEG in tetrahydrofuran (THF) (1 equiv) ($M_n = 1450$ Da) in the presence of DCC and DMAP to give the one free –OH

Figure 1. (a, b) $^1$H NMR spectra of Mono’s 1 and 2.
containing compound, 3, which had one free –OH of the PEG moiety (Figure S7). The free –OH group of compound 3 was functionalized with exo-Nor carboxylic acid in the presence of the reagents DCC and DMAP. The product was precipitated in cold hexane to obtain the boc-protected Nor-PEG amine (4) (Figure S8). Deprotection of compound 4 was carried out using trifluoroacetic acid. The formation of product 5 was confirmed by the disappearance of the δ = 1.39 ppm peak (Figure S9), the characteristic peak of the boc methyl proton.

After successful synthesis of amine-terminated Nor-PEG (5), Btn was reacted to it in the presence of the reagents DCC and DMAP (Scheme 1) to get Nor-PEG-Btn (Mono 3), which was highly water soluble. The formation of the product was characterized by an 1H NMR spectrum (Figure S10), in which all of the characteristic peaks appeared. The formation of Mono 3 was also confirmed by the matrix-assisted laser desorption ionization (MALDI) spectroscopy technique (Figure S18). After the successful synthesis of all monomers (Scheme 1; Figures 1 and S1–S10), the livingness conditions of all three monomers were explored. The linear increase in the molecular weight (Mn) of the homopolymers with increasing monomer/initiator ratio confirms the livingness (Figure 2a).

After confirming the livingness of all three monomers, the copolymerization condition was explored (Scheme 2).18,21,22 The monomers (Mono's 1–3) were dissolved in dry dichloromethane (DCM) and MeOH in a 9:1 ratio. A desired

Figure 2. (a) Mₙ vs M/I plot of Mono’s 1–3 and (b) gel-permeation chromatography (GPC) chromatogram of the final copolymer (Nor-Chl-Py-Btn).

Figure 3. Measurement of the CAC of Nor-Chl-Py-Btn using Nile red as a probe.

Scheme 2. Synthesis of the Copolymer (Nor-Chl-Py-Btn)
amount of second-generation Grubbs’ catalyst (G-2) was added in a two-necked flask, flushed with nitrogen, and dissolved in a minimum amount of anhydrous DCM (1 mL); this was then added to the flask containing the monomers. The monomer ratio for the copolymer synthesis was 1:1:1.5 mol equiv (Mono 1/Mono 2/Mono 3). The reaction was allowed to occur at room temperature, with stirring, until the polymerization was complete. After completion of the reaction, ethyl vinyl ether (0.5 mL) was added to quench the polymerization. An aliquot was taken for GPC analysis, and the remaining product was precipitated from ether, dissolved in THF again, passed through neutral alumina, and re-precipitated from ether.

The molecular weight of the copolymer was measured on a GPC instrument using a polymethyl methacrylate standard ($M_n = 26,000$, polydispersity index ($PDI) = 1.11$) (Figure 2b). After demonstrating successful synthesis of the copolymer (Nor−Chl−Py−Btn), the critical aggregation concentration (CAC) was calculated by measuring the fluorescence intensity of Nile red at different polymeric concentrations. The intensity was almost the same up to a certain polymeric concentration then

Figure 4. (a) DLS measurements of Nor−Chl−Py−Btn in water. (b) SEM images of Nor−Chl−Py−Btn. (c) TEM images of Nor−Chl−Py−Btn.

Figure 5. Emission spectra of 4-(1-pyrenyl) butyric acid (red), Mono 2 (green), and Nor−Chl−Py−Btn (blue) at the same concentration (10 μM).
an enhancement in the intensity confirmed dye encapsulation within the polymeric nanoaggregates. CAC was calculated by plotting the fluorescence intensity of Nile red at 636 nm against the polymeric concentration. The observed CAC value of Nor–Chl–Py–Btn was 1.2 μg/mL (Figure 3).

To measure the size of the newly designed nanoaggregates, dynamic light scattering (DLS) analysis was carried out under an aqueous environment. Because of the presence of large as well as complex amphiphilicity, Nor–Chl–Py–Btn was expected to self-assemble in the polar environment (Scheme 3).18,23,24

To confirm the aggregation of Nor–Chl–Py–Btn, DLS analysis was performed. The size of the aggregate was observed to be 90 nm, with a PDI of 0.35 (Figure 4a). To visualize the shape of the nanoaggregates, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed. From SEM and TEM (Figure 4b,c) analysis, the nanoaggregates were found to be spherical, with a size of about 90 nm, which was in good agreement with DLS measurements. The stability of the nanoaggregate system was confirmed by monitoring the particle size with respect to time. The particle size of Nor–Chl–Py–Btn in buffer (pH 7.4) was measured over intervals spanning days, and it was found that the particle size remained the same (Figure S12). After stabilization of the nanoaggregate in water, a photophysical study was carried out to authenticate its fluorescence property. The fluorescence emission spectrum of Mono 2 in DCM showed three characteristic monomer emission peaks at 375, 397, and 417 nm. The same was observed for 4-(1-pyrenyl) butyric acid as well. No stacking was observed at the monomeric level at micromolar (μM) concentrations. The fluorescence emission spectrum of Nor–Chl–Py–Btn in water showed three characteristic monomer emission peaks at 375, 397, and 417 nm and a featureless emission peak at 475 nm. The emission at 475 nm was attributed to the excimer emission of Py, which clearly indicated a strong interaction of the Py moiety in the excited state due to the constrained structure of the Nor backbone.25 Also, the emission under aqueous conditions established the hydrophobic core and PEG at the corona (Figure 5).

**RELEASE STUDY**

After proving the aggregation behavior of this unique copolymer, the release profile of the Nor–Chl–Py–Btn nanoaggregate was tested by dialysis studies (Figure 6).15 A dialysis bag containing a water solution of the final copolymer (5 mg/mL) was placed in a 50 mL beaker. The solution of the copolymer (Nor–Chl–Py–Btn) was dialyzed against a phosphate-buffered saline/acetonitrile mixture (pH = 5.5) to mimic the cancer cell pH. The absorbance of the buffer was measured at different time intervals up to 24 h.

The absorbance at 306 nm corresponded to Chl, whereas the absorbances at 282, 317, 333, and 352 nm were due to 4-(1-pyrenyl) butyric acid. The release study was performed for 24 h. It was interesting to note that at pH 5.5, Chl release was 68%, whereas the release of 4-(1-pyrenyl) butyric acid was 65% (Figure 6b). As the release kinetics of both 4-(1-pyrenyl) butyric acid and Chl were almost the same, we envisioned that the release profile at the cellular level could be easily observed by measuring the Py intensity.18 Finally, biocompatibility and cell viability studies of the newly developed nanoaggregate system were conducted.

**BIOLOGICAL STUDIES**

To reveal the importance of the Nor–Chl–Py–Btn nanoaggregate, three cell lines were considered: HeLa wild-type (wt) cells (human cervical cancer cell line), HEK 293 cells (human embryonic kidney cells), and MCF 7 cells (human breast cancer cell line). The cells were maintained in minimum essential medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) and were incubated at 37 °C in a 5% CO2 environment according to the ATCC recommendations. The cytotoxicity of the Nor–Chl–Py–Btn nanoaggregate on HeLa wt and HEK 293 cells was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. A fresh solution of MTT (20 μL) was added to each well, followed by incubation for 4 h in 5% CO2 at 37 °C.

From the cytotoxicity assay, it was clear that in the Btn receptor-positive (BR +ve) HeLa wt cells Nor–Chl–Py–Btn was more toxic than Chl (Figure 7c). Interestingly, the same experiment with HEK 293 cells, which are Btn receptor-negative (BR –ve), revealed that the cytotoxicity was more for Chl than that for Nor–Chl–Py–Btn (Figure 7a). A comparative statistical analysis of the cytotoxicity assay results clearly described that Nor–Chl–Py–Btn was better in the case of HeLa wt (BR +ve) cells than in the case of HEK 293 (BR –ve) cells (Figure 7b,d). It also revealed that the Nor–Chl–
Py−Btn nanoaggregate was not toxic to normal cell lines (HEK 293), as in the case of Chl (Figure 7b,d). This clearly suggested the importance of our design. The results also emphasized the importance of receptor-assisted internalization of the nanoaggregates. The cytotoxicity of the copolymer without drug was tested in three different cell lines (HEK 293, HeLa wt, MCF 7); it did not show a cytotoxic effect toward any of the cell lines, which confirms that the cytotoxic effect was due to the chemotherapeutic agent Chl (Figure S15). To confirm the site-specific nature of the nanoaggregate, the cytotoxicity toward another Btn receptor-positive cell line (MCF 7) was tested. It is clear that the nanoaggregate shows high toxicity toward MCF 7 cell lines as like HeLa wt (Btn-positive) compare to HEK 293 (Btn-negative), which further confirms the site-specific nature of our nanoaggregate (Figure S13).

An epifluorescence microscope was used to track Py emission in Nor−Chl−Py−Btn. As observed in Figure 8b, nanoaggregates incubated with HeLa wt cells showed an obviously higher fluorescence intensity than those incubated with HEK 293 cells at a given time and a 100 μg/mL concentration. This obvious high fluorescent intensity was due to Btn-assisted internalization. To further confirm the receptor-mediated internalization, a cellular uptake study of another Btn-positive cell line (MCF 7) was performed. High internalization of the nanoaggregate in MCF 7 (human breast cancer cell line) was observed, which further confirms the site-specific nature of the nanoaggregate (Figure S14).

As the cells were not stained with DAPI, the observed blue expression under the epifluorescence microscope was only from the Py motifs. This clearly suggested the significance of our design. As both Chl and Py were linked to the polymer via an ester backbone, their release kinetics as well as concentrations could be directly related (Figure 6). From the epifluorescence microscope live-cell image analysis, it was observed that the intensity of the HEK 293 cell line was less than that of the HeLa wt cell line. It was very clear from the results for the HeLa wt cell line that the intensity was very prominent because of the BR +ve nature of this cell line, that clearly confirmed the greater internalization of the nanoaggregates into HeLa wt cell line was due to the presence of btn receptor. Flow cytometry results revealed that the fold change in the HeLa wt (BR +ve) cell line was 2.33 and 3.58 times higher at 50 and 100 μg/mL concentrations, respectively, than that in the HEK 293 cell line (BR −ve) (Figures 8c and 9). From the above cell viability assay, epifluorescence microscope analysis, and flow cytometry results, it clearly established that the Nor−Chl−Py−Btn nanoaggregate is a potential site-specific theranostic agent for the purpose of drug delivery as well as imaging.

### CONCLUSIONS

In conclusion, with the ability to provide concurrent therapy and fluorescence imaging, the Nor−Chl−Py−Btn nanoaggregate has great potential and applicability in the field of medicine as a nanotheranostic agent. Compared to physical...
encapsulation, chemical conjugation of both the drug and imaging motif with the same linker helps track the non-fluorescent drug in cell in vitro studies. The inclusion of Btn motif to the polymeric backbone results in enhanced tumor accumulation due to the site-specific nature. We envision that the newly designed nanotheranostic agent, nanoaggregate Nor−Chl−Py−Btn, has potential application in personalized treatment via simultaneous treatment and monitoring.

## EXPERIMENTAL SECTION

### Synthesis of Mono 1.
Chl (250 mg; 1.2 mmol) was dissolved in dry DCM in a round-bottom flask, and 285 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (1.48 mmol) was added to it. The reaction mixture was stirred for 1 h, and then, 1 was added to it along with DMAP. Thereafter, the reaction mixture was stirred for 24 h. After completion of the reaction, the reaction mixture was washed with saturated NaHCO3. The DCM layer was dried over anhydrous Na2SO4 and evaporated to obtain the pure product as a yellow oil. Yield: 470 mg (60%, 0.96 mmol)

1H NMR (DMSO-d6, 500 MHz): δ (ppm): 7.01 (m, 2H), 6.6 (q, 2H), 6.3 (d, 2H), 4.1 (t, 2H), 3.65 (8H, broad), 3.1 (m, 2H), 2.9 (t, 2H), 2.6 (s, 2H), 1.23−1.34 (m, 2H). 13C NMR (DMSO-d6, 500 MHz): 178, 172, 145, 137.5, 129, 111, 60, 57, 52, 47, 45, 42, 40.5, 37, 33, 26 (Figures 1 and S3). ESIMS: [M + Na]+ calcd for C25H30Cl2N2O4, 515.16; observed, 515.14 (Figure S16).

### Synthesis of Mono 2.
Pyrene butyric acid (950 mg; 3.29 mmol) was dissolved in dry DCM. DCC (850 mg; 4.08 mmol) was added to it and stirred for 1 h. Then, 650 mg (3.14 mmol) of 1 and 50 mg of DMAP were added to it and stirred for 24 h. Following this, the reaction mixture was filtered and the filtrate was washed with water and then concentrated. Column chromatography separation was performed to obtain the pure product as a white powder. Yield: 1 g (70%, 2.3 mmol)

1H NMR (DMSO-d6, 500 MHz): δ (ppm): 8.35 (d, 1H), 8.25 (m, 2H), 8.21 (q, 2H), 8.12 (d, 2H), 8.05 (t, 1H), 7.9 (d, 1H), 6.1 (t, 2H), 4.1 (t, 2H), 3.6 (t, 2H), 2.84 (t, 2H), 2.55 (d, 2H), 2.36 (t, 2H), 1.97 (m, 2H), 1.03−1.08 (m, 2H). 13C NMR (DMSO-d6, 500 MHz): δ (ppm): 177, 172, 137.5, 136, 130.8, 130.4, 129.3, 128.1, 127.5, 128.2, 126.5, 126.1, 125, 124.8, 124.2, 124.1, 123.5, 60, 47.1, 44.3, 42.1, 37, 33, 31.8, 26.5 (Figures 1b and S4). ESIMS (m/z): calcd for C31H27NO4, 477.19; observed, 477.216 (Figure S17).

### Synthesis of Mono 3.
Btn (100 mg; 0.409 mmol) was dissolved in dry dimethylformamide and 70 mg (0.451 mmol) of EDC·HCl was added to it. The reaction mixture was stirred for 1 h. Nor−PEG-amine (650 mg; 0.391 mmol) and DMAP (50 mg) were added to it. The reaction mixture was stirred for 24 h. After completion of the reaction, the product was precipitated in cold hexane three times to obtain the pure product as a white sticky mass. Yield: 500 mg (70%, 0.273 mmol)

1H NMR (DMSO-d6, 500 MHz): 8.2 (d, 1H), 6.9 (d, 1H) 6.2 (d, 2H), 4.59 (t, 2H), 4.43−4.4 (m, 2H), 3.6 (s, broad), 2.9 (s, 2H), 2.19−2.28 (m, 2H), 2.30−2.33 (t, 2H), 2.0−2.07 (m, 2H), 1.86−1.94 (m, 2H), 1.50−1.56 (m, 2H), 1.2−1.6 (m, 2H) (Figure S10). The formation of the product was also confirmed by the MALDI spectrum (Figure S18).

### Polymerization Procedure.
After successful synthesis of the monomers, the homopolymerization conditions were explored. To assess the livingness of the monomers (Mono’s 1−3), different amounts of Grubbs’ second-generation catalyst were added to each monomer dissolved in anhydrous dichloromethane (CH2Cl2) and stirred under a nitrogen atmosphere for 1 h, for Mono’s 1 and 2, or 6 h, for Mono 3. The polymerization reaction was carried out inside a glove box. After completion of the reaction, an aliquot was taken out for GPC analysis, and the remaining portion was quenched

![Figure 8](image-url)
with ethyl vinyl ether and precipitated from diethyl ether three times. The different molecular weights of the homopolymers of all three monomers were measured by the GPC technique and plotted against $M/I$ (Figure 2a).

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.6b00167.

Experimental details for all monomers and NMR characterization of all compounds; mass of all final monomers (PDF)

Live-cell images of the cellular uptake at different time intervals in the HEK 293 and HeLa wt cell lines (AVI) (AVI) (AVI) (AVI)

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Notes
The authors declare no competing financial interest.

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