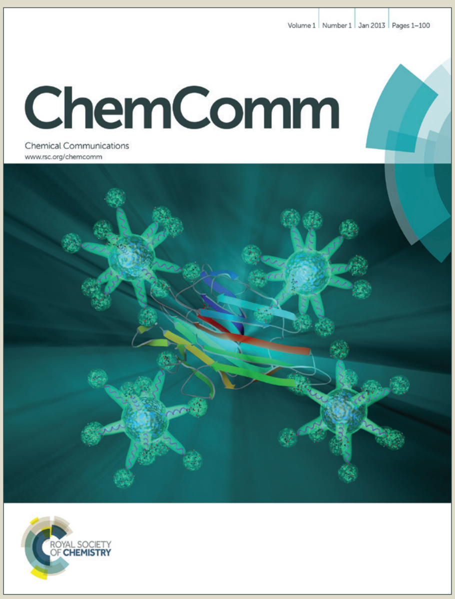
Volume 1 | Number 1 | Jan 2013 | Pages 1–100
ChemComm
Chemical Communications
www.rsc.org/chemcomm
ROYAL SOCIETY OF CHEMISTRY

Journal Name ChemComm Dynamic Article Links ▶

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Efficient approach to prepare multiple chemotherapeutic agents conjugated nanocarrier

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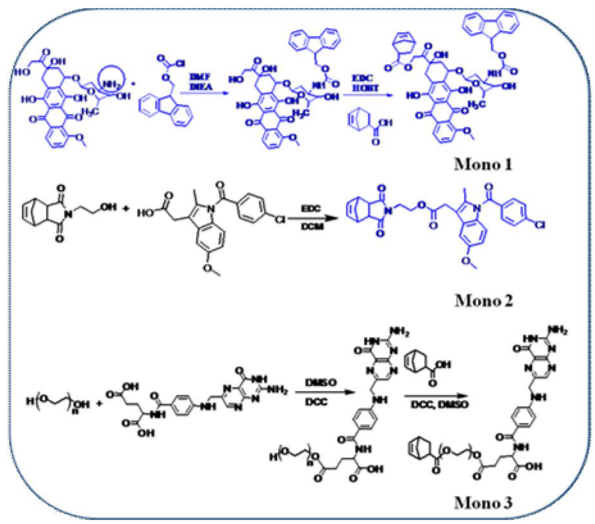
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

pH responsive, multiple chemotherapeutic agents derived nanocarrier has been synthesized by conjugating doxorubicin, indomethacin, and folate to the backbone of norbornene polymer. Drug molecules are connected to the norbornene backbone by ester linker to demonstrate the pH responsive capabilities. The complete chemical and biological properties of new norbornene-based polymeric nanocarrier, intended for combination cancer chemotherapy, are discussed.

Drug targeting nano-carriers are developed for improving biodistribution of therapeutic drugs.[1a-c] Many anticancer drugs such as paclitaxel, doxorubicin **(DOX)**, camptothecin, chlorambucil (**CHO**) and indomethacin (**IND**), have shown improved pharmacokinetic profiles and clinical efficacy following polymer conjugation.[2] The EPR effect makes polymers remain in the body more time compared with the monomeric drugs.[3a-c] Polymeric carriers are well established as a delivery vehicle for a single therapeutic agent,[4] but very recently they have been employed to the delivery of multi-agent therapy.[5a,b] Drug resistance could be achieved using combined therapy of two or more drugs.[6] Multi-agent therapy can reduce the chemo-resistance, which has been practiced as a primary cancer therapy.[7] Promising results have been obtained on the 'Co-administration' regimens: For example, Pegylated liposomal doxorubicin, has been successfully combined with gemcitabine.[8] Besides this, 'Co-formulating' chemotherapeutic approach supports a model where two different pharmacologically active agents are delivered to the site simultaneously.[9] Despite the fact that combination therapy often improves therapeutic efficiency in cancer treatment, it is unusual that cocktail of drugs is still a largely unexplored area.[10] It remains a huge challenge for researchers working in the combination therapy to come up with a smart and efficient approach to reduce side effects while keeping the pharmacokinetics, and biodirstubutions.[11]

Herein, we report an efficient method to prepare a pH responsive smart nanocarrier with multifunctional drugs, doxorubicin-indomethacin drug cocktails along with folic acid **(FOL)** for site specific drug delivery. All the three functionalities, namely, **DOX, IND, & FOL** are delicately conjugated to the norbornene backbone via ester linkers to produce novel monomers. For the preparation of proposed nanocarrier, we have utilized living ring-opening metathesis polymerization (ROMP).[12a-e] The primary attractive reason for the option of ROMP to synthesize mono disperse polymeric prodrugs is for its exceptional functional group tolerance. Grubbs' second generation catalyst is used for all the polymerization reactions. To best of our knowledge this is a simple and efficient method to conjugate the complex structure and deliver it successfully under the mild acidic conditions.



Scheme 1: Synthesis of monomers **Mono 1-3.**

Towards the motivation of making a combination therapy, monomers namely, **NOR-DOX (mono 1), NOR-IND (mono 2), NOR-PEG-FOL (mono 3)** were synthesized (**Scheme 1**). The detailed synthetic procedure and complete characterization of these monomers are discussed in the (**Figure** S1-S15). The synthetic importance of the design was strongly encouraged by the freely water soluble nature of **mono-3** (**Scheme 1**). Due to this, we envisioned that there would not be a need for separate PEG polymer segment to make the system water soluble.[3b] Next, homopolymerization of **mono 1 (Scheme S1)** was carried out by using second generation Grubbs' catalyst, at room temperature in dry DCM and Methanol (9:1 v/v %) solvent system. New signals were observed at 5.0-5.4 ppm and norbornene olefinic protons were disappeared at 6.10-6.14 ppm, indicating the formation of the polymer. The polymerization was monitored by ^{1}H NMR spectroscopy. The observed molecular weight (Mn) and polydispersity index (PDI) from the GPC analysis that suggested the polymerization of mono **1** was done in a very controlled fashion. Similarly, the homopolymerization of **mono 2, (Figure**

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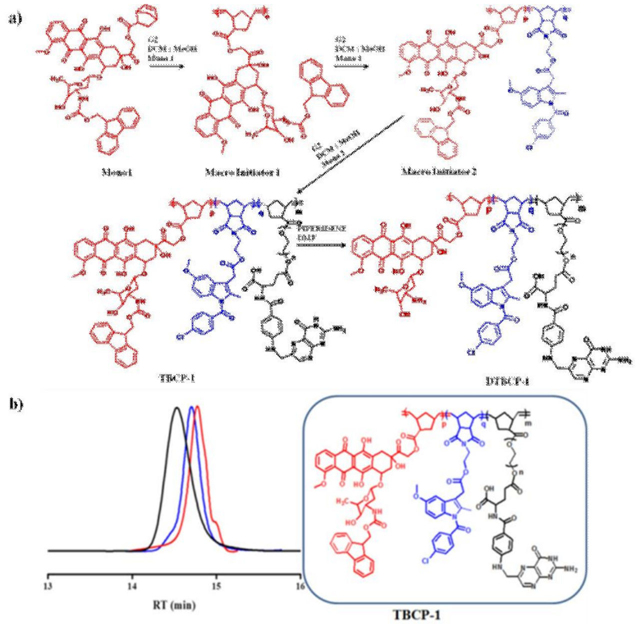
†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

S16), and homopolymer of **mono 3** were done to produce well defined polymers (**Scheme S1**).

After establishing the polymerization conditions for all the monomers, the triblock copolymerization **(Figure 1c)** was carried out using second generation Grubbs' catalyst, at room temperature in dry DCM and Methanol (9:1 v/v %) solvent system by sequential addition of **mono1, 2 and 3 respectively** to get the triblock copolymer **TBCP-1**. The polymerisation was monitored by ^{1}H-NMR spectroscopy. The molecular weights of the macro initiator **1** (NOR-**FMOC - DOX**, Mn = 10000, PDI= 1.1), macro initiator **2** (NOR-**FMOC - DOX-IND**, Mn = 16000, PDI= 1.2) and the final triblock copolymer (**TBCP-1**), Mn = 29000, PDI = 1.2) were measured by GPC using polystyrene



Triblock Copolymer	P	m	n	P	m	n	Mole Ratio (p/m/n)	Mn,block th (g/mol)	Mn,block (GPC) (g/mol)	PDI
TBCP-1	15	15	15	11	8	7	0.1133	37000	29000	1.20

GPC analysis of TBCP-1 (M_n = 29 kDa; PDI = 1.20), macroinitiator 2 (M_n =17 kDa; PDI = 1.1), macroinitiator 1 (M_n = 10 kDa; PDI = 1.1).

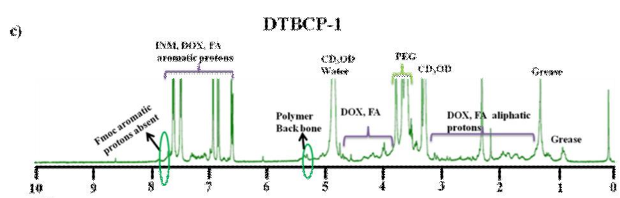


Figure 1. a) Synthesis of **DTBCP-1** b) GPC traces of **TBCP-1 c) ^{1}H NMR** spectrum of **DTBCP-1.**

standards and the results are shown in the **Figure 1b**. The shifting in the GPC traces in **Figure 1b** clearly indicated the formation of triblock copolymer, **TBCP-1.** Also the formation of **TBCP-1** was conformed through ^{1}H-NMR and FTIR spectroscopy. The disappearance of norbornene olefinic protons at 6.2 ppm and the formation of new signals at 5.0 - 5.3 ppm, confirmed the polymerization. In addition, all the characteristics signals for **DOX, IND** and **FOL** functionalities were also observed in the final triblock copolymer (**Figure 1c**). Next de-protection of Fmoc group in triblock copolymer **TBCP-1** was carried **(Figure 1a)** by using piperidine.[13] The deprotected copolymer was isolated by using cold diethyl ether. The disappearance of aromatic protons in the ^{1}H-NMR spectroscopy confirmed the deprotection (**Figure 1c).** After confirming the formation **DTBCP-1** triblock copolymer thoroughly, aggregation behaviour of the copolymer was studied in the aqueous/polar conditions. Due to the presence of huge amphiphilicity, the polymer was expected to self-assemble in the polar environment. To prove the aggregation behaviour of **DTBCP-1**, dynamic light scattering (DLS) analysis was performed. To perform DLS experiment, 1 mg of **DTBCP-1** was dissolved in 10 mL of methanol and stirred for 1 h to ensure the complete polymer solubilisation. From this solution, 2.5 mL of sample was taken in a vial to which 18% of water was added under constant stirring and the particle size was measured (**Figure 2b**). The size of the aggregates was about 280 nm with 0.21 PDI. The unimodel distribution observed in the DLS suggested the formation of the uniform nano-aggregates.

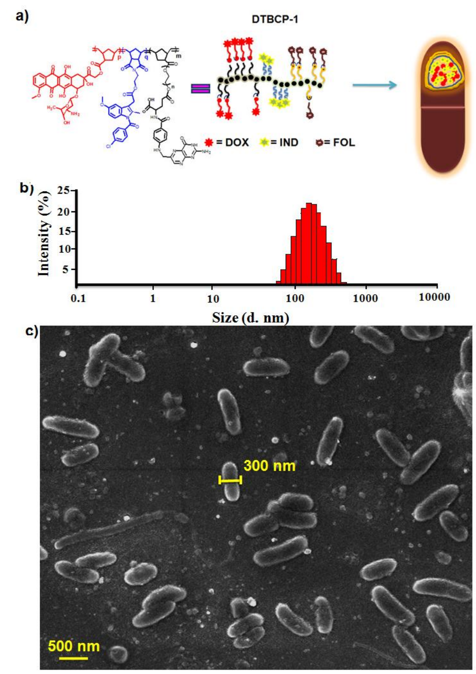


Figure 2. a) Cartoon representation for self assembly of **DTBCP-1. (b)** DLS measurement of **DTBCP-1** aggregates in aqueous solution**;** c) SEM image of **DTBCP-1** aggregates spin coated on a silicon surface.

To find out the shape of the nano-aggregates, scanning electron microscope (SEM) and transmission electron microscope (TEM) were performed. From SEM and TEM (**Figure 2c** and **S18**) analysis, the particle size was measured to be about 300 nm, which was in good agreement with DLS measurements. It was interesting to note from the SEM analysis that the observed aggregates from **DTBCP-1** were capsule-like in shape and assembled in controlled manner (**Figure 2c** and **S18**). This encouraged us to propose the cartoon representation for the nanocarrier **(Figure 2a)**. Based on the shape, we hypothesized that under the aqueous and physiological environment, the nanocarrier self assembled into capsule-like aggregates. We also envisioned that the relatively polar **FOL** motifs would be in the corona whereas hydrophobic **DOX** and **IND** would occupy the core of the aggregates **(Figure 2a)**. Having proven the aggregation behaviour of these unique triblock copolymers, the reservoir capabilities were tested by doing dialysis studies (supporting information). It was observed that after 12 hrs, there was no significant increase in the intensity of absorption. It was observed that at pH 5.5, **DOX** release was observed 64% and release of indomethacin 60% (**Figure 3a & Figure S17**).

Finally, the biocompatibility as well as the cell viability studies of the newly developed multi-drug nanocarrier system was explored. The cell viability experiment was done in 4T cell lines. The effect on the cell viability was evaluated by incubating it with the increasing concentration of **COPY-DOX**[3a,12b] (control)**, DTBCP-1** (25 µg/mL to 200 µg/mL) up to 24 h, after which the viability of the cell was determined by MTT assay (**Figure 3c**). The effect on cell growth and cell division were observed as response. It was observed that at 200 µg/mL concentration, **DTBCP-1** was appeared to be more toxic to the 4T cell line in comparison with the control molecule.

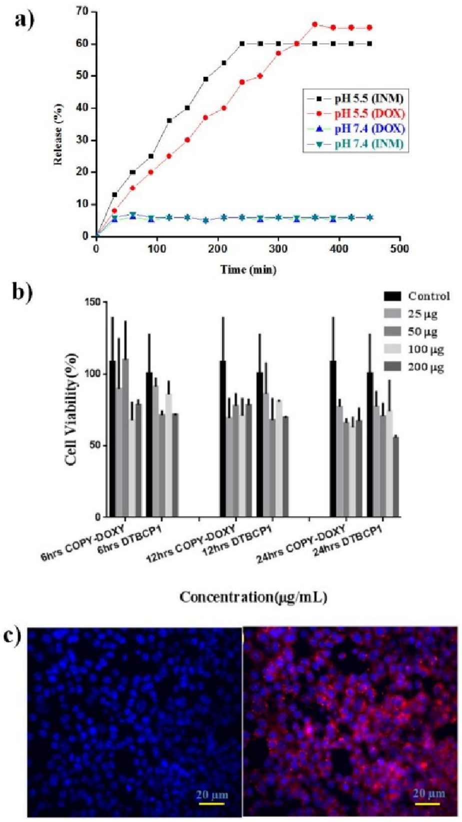


Figure 3. a) Drug release studies of **DTBCP-1** b) Cell viability studies of **DTBCP-1** on 4T cells c) Confocal Laser Scanning Microscopy (CLSM) studies on, **DTBCP-1** in 4T cells.

From the cell viability, it was observed that the effectiveness of having combination of drugs (**DTBCP-1**) was greater in comparison to the only one drug conjugate to the same polymer (**COPY-DOX**).[3a, 12b] A control experiment with different ratios of doxorubicin or indomethacin produced different degree of cytotoxicity (**Figure S19**). To demonstrate the increased cell viability was only due to the unique design but not due to any other impurities, for example, residual Ru metal from the Grubbs' catalyst, a control experiment was performed on 4T cells. Homopolymers of norbornene (**Figure S18**a), and norbornene PEG (**Figure S20b**) were chosen as control molecules. It was interesting to note that cells were normal even at very high concentrations of these polymers (250 µg) which clearly confirmed that the residual Ru from the catalyst was not responsible for the observed toxicity. Obviously the observed toxicity was due to the release from the drug molecules of **DTBCP-1** (**Figure S20c).**

Nanocarrier internalization was studied using CLSM. The pH of the 4T cells was more acidic, so the release of **DOX** from **DTBCP-1** was more pronounced in 4T cells (**Figure 3b**). The known acidic environment formed during endosomal uptake process, would induce **DOX** release from the **DTBCP-1** in 4T cells. Increasing the incubation time did not change the rate or amount of internalization (data not shown) and it was noted that the amount of release of drugs from the nanocarrier was highest in 4T cells due to its relatively more acidic nature compared to other cell lines. It was very interesting observation to note as it clearly emphasized the importance of our design of **DTBCP-1** with ester linker. It should be noted that **IND** motifs were not emissive in nature, release of these motifs was not observed in the confocal studies. Since all the drugs were attached through ester linker, their release profile was also assumed to be similar to the **DOX** release. Finally, to prove the different degree of cytotoxicity as the function of different ratios of **DOX** and **IND**, flow cytometery experiments were performed with different molecular weights of **DTBCP-1**. It was very clear from the results that more the number of **DOX** and **IND** motifs, greater was the cytotoxocity (**Figure SI21**).

Conclusions

In conclusion, norbornene based triblock copolymers conjugated with multi-anticancer drug cocktails (**DTBCP-1)** have been prepared using ROMP technique. This smart nanocarrier (**DTBCP-1)** is compact enough to meet the size requirement for a drug cargo as all the components can be self-contained with in 80 nm. We have demonstrated that the drugs can be elegantly conjugated to the norbornene backbone and efficiently delivered from the self-assembled nanocarrier in mild acidic conditions. The enhanced anticancer activity is expected for the newly designed nanocarrier due to the multi-drug delivery. Presence of folic acid **(FOL)** motif adds the value of the nanocarrier to act as new dual-sensitive tumour targeting drug delivering materials. Our unique design can open up a new avenue for a more effective cancer therapy through well informed decision-making.

Notes and references:

NVK thanks CSIR for the research fellowship. HD thanks DST. RS thanks DST, and DBT, New Delhi for Ramanujan Fellowship. RS and JDS thank IISER-Kolkata for providing the infrastructure and start up funding.

1. (a) R. Duncan, *Nat. Rev. Cancer*., 2006, **6**, 688; (b) R. Duncan, *Nat. Rev. Drug Discovery*., 2003, **2**, 347.
2. R. Duncan, *Anti-Cancer Drugs.,* 1992, **3**, 175.
3. (a)V. N. Rao, S. R. Mane, A. Kishore, J. Das Sarma and R. Shunmugam, *Biomacromolecules,* 2012, **13**, 221. (b) V.N. Rao, Naidu, S. Sarkar, J. Das Sarma, R. Shunmugam, *Bioconjugate Chem.,* 2014, **25**, 276; (c) A. K. Singla, A. Garg and D. Aggarwal, *Int. J. Pharm.,* 2002, **235**, 179.
4. K. L. Christman, R. M. Broyer, E. Schopf, C. Kolodziej, Y. Chen and H. D. Maynard, *Langmuir,* 2011, **27**, 1415.
5. (a)X. R. Song, Z. Cai, Y. Zheng, G. He, F. F. Y. Cui, D. Q . Gong, S. X. Hou, S. J. Xiong, X. J. Lei and Y. Q. Wei, *Eur. J. Pharm. Sci.,* 2009, **37**, 300; (b) F. Ahmed, R. I. Pakunlu, A. Brannan, F. Bates, T. Minko and D. E. Discher, *J. Controlled Release.,* 2006, **116**, 150.
6. Y. Huang, C. M. Horvath and S. Waxman, *Cancer Res.,* 2000, **60**, 3200.
7. Y. Bae, T. A. Diezi, A. Zhao and G. S. Kwon, *J. Controlled Release.,* 2007, **122**, 324.
8. T. Lammers, V. Subr, K. Ulbrich, P. Peschke, P. E. Huber, W. E. Hennink and G. Storm, *Biomaterials,* 2009, **30**, 3466.
9. Y. Bae, T. A. Diezi, A. Zhao and G. S. Kwon, *J. Control. Release.,* 2007, **122**, 324.
10. K. J. Watson, S. J. Park, J. H. Im and S. T. Nguyen, *Macromolecules*, 2001, **34**, 3507.
11. (a)N. Kolishetti, S. Dhar, P. M. Valencia, L. Q. Lin, R. Karnik, S. J. Lippard, R. Langer and O. C. Farokhzad, *Proc. Natl. Acad. Sci.,* 2010, **107**, 17939; (b) S. Aryal, C. M. Hu and L. Zhang, *Small,* 2010, **6**, 1442.
12. (a) V. N. Rao, A. Kishore, S. Sarkar J. Das Sarma and R. Shunmugam, *Biomacromolecules* 2012, **13,** 2933; (b) K. J. Watson, S. J. Park, J. H. Im and S. T. Nguyen, *Macromolecules*, 2001, **34**, 3507; (c) K. H. Mortell, M. Gingras and L. L. Kiessling, *J. Am. Chem. Soc.,* 1994, **116**, 12053; (d) H. D. Maynard, Y. O. Sheldon and R. H. Grubbs, *Macromolecules,* 2000, **33,** 6239.

Table of Content Graphics:

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