CHEMISTRY
A European Journal

A Journal of
ChemPubSoc Europe

Accepted Article

Title: Supramolecular Self-Assembly of Histidine-Capped-Dialkoxy-Anthracene: A Visible Light Triggered Platform for facile siRNA Delivery

Authors: Sachin Patil; Basem Moosa; Shahad Alsaiari; Kholod Alamoudi; Aws Alshamsan; Abdulaziz Almailk; Karim Adil; Mohamed Eddaoudi; Niveen Khashab

To be cited as: Chem. Eur. J. 10.1002/chem.201601442

Link to VoR: http://dx.doi.org/10.1002/chem.201601442

Supported by
ACES

WILEY-VCH

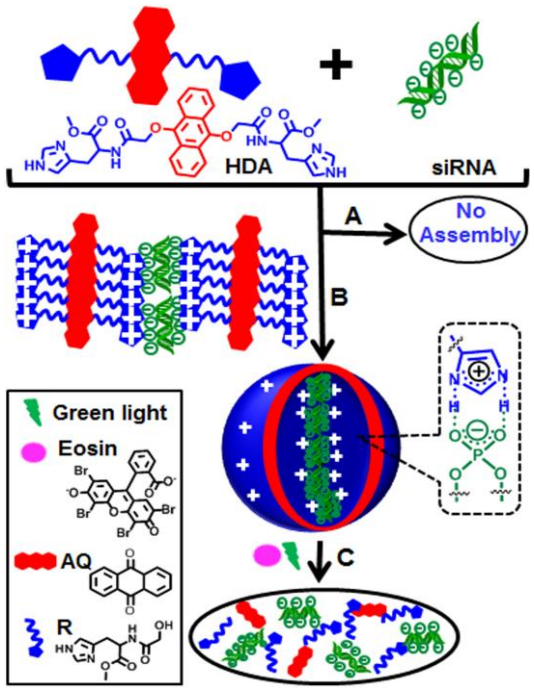
Supramolecular Self-Assembly of Histidine-Capped-Dialkoxy-Anthracene: A Visible Light Triggered Platform for facile siRNA Delivery

Sachin P. Patil, Basem A. Moosa, Shahad Alsaiari, Kholod Alamoudi, Aws Alshamsan, Abdulaziz AlMalik, Karim Adil, Mohamed Eddaoudi, and Niveen M. Khashab*

ABSTRACT: Supramolecular self-assembly of histidine-capped-dialkoxy-anthracene (HDA) results in the formation of light responsive nanostructures. Single-crystal X-ray diffraction analysis of HDA shows two types of hydrogen bonding. The first hydrogen bond is established between the imidazole moieties while the second involves the oxygen atom of one amide group and the hydrogen atom of a second amide group. When protonated in acidic aqueous media, HDA successfully complexes siRNA yielding spherical nanostructures. This biocompatible platform controllably delivers siRNA with high efficacy upon visible light irradiation leading up to 90% of gene silencing in live cells.

Self-assembly of macromolecules or small molecules through non-covalent bonds to generate supramolecular complexes is a prevalent process in nature. From a research perspective, this principle is commonly used to build up supramolecular nano- and microstructures that are developed for a variety of applications including drug/ gene delivery, cell imaging, and tissue regeneration.[1] Self-assembly in water has received major attention because it can generate dynamic materials starting from versatile building blocks such as block copolymers, peptides, liposomes, and dendrimers.[2] Such assemblies are mainly reported in the form of supramolecular hydrogels or host-guest complexes based on macrocyclic receptors.[3] These systems are also stimuli responsive by nature and can be easily tailored to respond to a variety of triggers including pH, heat, light, enzymes, and redox.[4] Among a plethora of stimuli, light is a clean, easy to control, and low cost trigger that can be easily applied.[5] The known majority of light-responsive scaffolds such as azobenzene, o-nitrobenzyl and spiropyran, are responsive to ultraviolet irradiation.[6] Unfortunately, the high-energy light (~6 eV) is harmful and can hardly penetrate tissues, which hampers their potential use in clinical settings.

Visible light responsive (VLR) moieties such as 9-alkoxyanthracene, 9,10- dialkoxyanthracene (DA), amino acrylate and cis-oxo- or thioether were developed as a safer alternative with an improved penetration capability.[7] VLR systems can also be coupled with nano-transducers to covert deeply penetrating near-infrared light to visible light.[8] Specifically, DA group showed favorable green light responsiveness in the presence of singlet oxygen, which can cleave DA derivatives into 9,10-anthraquinone (AQ). Despite the remarkable progress made in the design and assembly of VLR polymeric[9] and host-guest[10] assemblies, a self-organization-based small molecule VLR assembly has, so far, proved to be elusive. Herein, supramolecular self-assembly of histidine-capped-9,10-dialkoxy-anthracene (HDA) to form visible light responsive platform is reported. Single-crystal X-ray diffraction analysis of HDA shows two types of hydrogen bonding in the assembly comprising imidazole and amide moieties.



Scheme 1. Schematic representation: (A) No assembly of HDA with siRNA at pH 7.36; (B) Formation of spherical nanostructures of HDA with siRNA at pH 6; (C) siRNA-HDA complex disassembled after green light exposure in the presence of eosin (visible light sensitizer).

[a] Dr. S. P. Patil[†], Dr. B. A. Moosa[†], S. Alsaiari, K. Alamoudi, Prof. N. M. Khashab
Smart Hybrid Materials (SHMs) Laboratory,
King Abdullah University of Science and Technology (KAUST),
Thuwal, Makkah 23955-6900, Kingdom of Saudi Arabia.
Dr. Aws Alshamsan
Department of Pharmaceutics, College of Pharmacy and King Abdullah Institute of Nanotechnology, King Saud University, KSA
Dr. Abdulaziz Almalik
Center of Excellence in Nanomedicine, King Abdulaziz City for Science and Technology (KACST), Riyadh, KSA
Dr. K. Adil, Prof. M. Eddaoudi,
Functional Materials Design, Discovery and Development Research
King Abdullah University of Science and Technology(KAUST),
Thuwal, Makkah 23955-6900, Kingdom of Saudi Arabia.
[†] These authors contributed equally.
Supporting information for this article is given via a link at the end of the document.

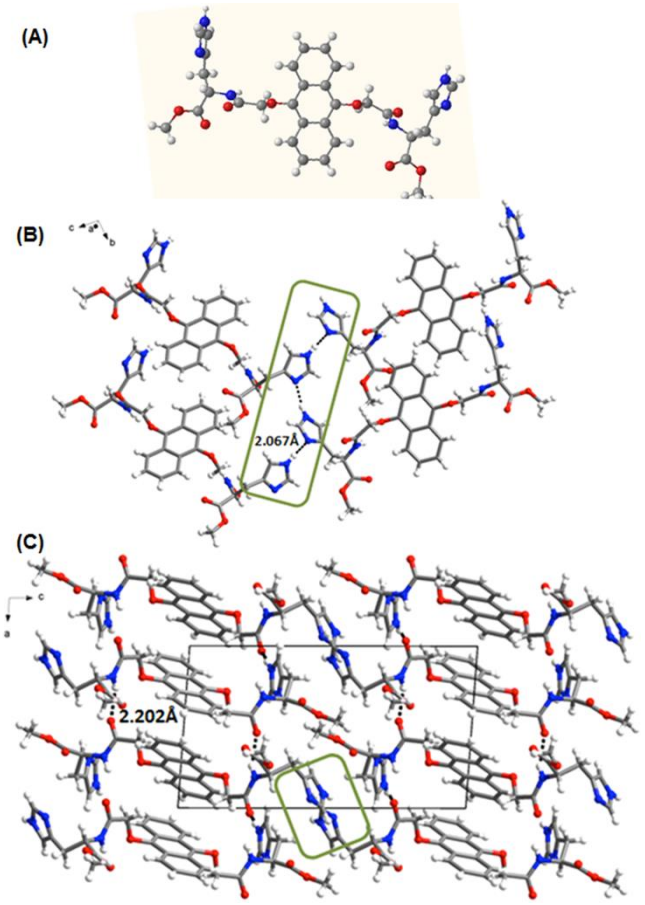


Figure 1. Crystal structure of (A) HDA; (B) Hydrogen bonding between imidazole moieties (2.067 Å); (C) HDA along the b axis showing hydrogen bonding between amide moieties (2.202 Å).

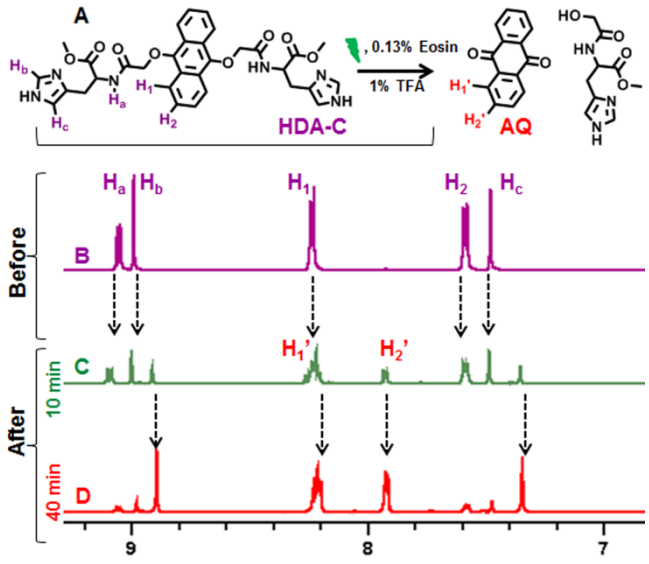


Figure 2. (A) Photolysis of HDA; Aromatic region of ^{1}HNMR spectra (DMSO-d_6) of (B) HDA-Eosin-CF_3COOH (HDA-C); (C) after 10 min green light exposure; (D) after 40 min green light exposure.

When protonated in water at pH 6, this supramolecular assembly can efficiently complex siRNA forming spherical nanostructures, whereas no complexation could be achieved at neutral conditions (pH 7.36) (Scheme 1). Electrostatic interaction between the positively charged HDA and negatively charged RNA is the major driving force of this assembly where we hypothize that the two protonated HDA arms are curving to complex RNA and thus yielding spherical nanostructures. Upon green light irradiation in the presence of eosin (photosensitizer), the spherical nanostructures disassembled releasing the trapped siRNA (Scheme 1). To the best of our knowledge, this is the first example of VLR small molecule self-assembly for externally controlled siRNA delivery. The histidine moiety not only provides a positive corona for electrostatically binding siRNA but also proves effective in improving siRNA efficacy by promoting endosomal escape.[11]

Fluorescent HDA was synthesized starting from non-fluorescent anthraquinone (AQ) (Scheme S1). The ^{1}H, and ^{13}C spectra of HDA revealed the formation of a pure organic building block. Colorless block single crystals of HDA were obtained by recrystallization from *N,N*-dimethyl formamide. Single-crystal X-ray diffraction (SCXRD) analysis showed that HDA crystallized in monoclinic space group $P2_1$ (Table S1). Figure 1 shows the packing of molecules through two types of hydrogen bonding. The first hydrogen bond (2.067(1) Å) occurs between imidazole moieties (Figure 1B) while the second (2.202(1) Å) involves the oxygen atom of one amide group and the hydrogen atom of a second amide group (Figure 1C).

The photolysis of HDA was performed and monitored via NMR spectroscopy (Figure 2 and S1). All ^{1}HNMR measurements were done in DMSO-d_6 instead of D_2O as unwanted intermolecular aggregation drastically broadens the signals making them unidentifiable. AQ is the major product of visible light decomposition of HDA-composite (HDA-C) which contains HDA with 1 mol % of eosin and 1% of TFA (Figure 2A). Proton peaks of HDA-C (H_a–H_h; Figure 2B and S1B) shifted downfield compared to pure HDA (Figure S1A) due to the presence of TFA. Upon green light irradiation (>500 nm for 10 min.), HDA-C peaks started to decrease while new peaks that are assigned to AQ (H_1'–H_2') were observed (Figure 2C and S1C–E). Increasing light exposure duration to 40 mins resulted in more prominent AQ peaks, which supports the successful photolysis of HDA (Figure 2D and S1D).

Transmission electron microscopy (TEM) was employed to further study HDA assembly (Figure S2). HDA self-assembled into uniformly dispersed nanorods (30–60 nm width and 90–120 nm height; Figure S2A). Upon visible light exposure (40 mins), the rod-like nanostructures disappeared and random fragments were observed (Figure S2B).

All self-assembly studies were originally conducted at neutral conditions however, protonation of the imidazole moieties at pH 6 increased the charge of the assembly to +39 compared to -11 at pH 7.36 (Figure S3). This pH-driven charge reversal prompted us to test this assembly for encapsulation of negatively charged cargo such as siRNA. The RNA interference machinery can be exploited to silence nearly any gene in the body, giving it a very broad therapeutic potential. However, naked siRNA is subject to degradation by endogenous enzymes, and is too large and too negatively charged to cross cellular membranes.[12] A variety of

materials have been explored to package siRNA including, liposomes, polymeric systems, and inorganic nanoparticles.[13] However, these "conventional" systems are heterogeneous in size, composition, and surface chemistry, which can lead to substandard performance, low efficacy, and possible toxicity. Thus, self-assembled small molecules can potentially succeed in siRNA delivery.[14] Interestingly, HDA readily complexed siRNA and assembled into stable spherical nanostructures (≈500 nm; Figure 3A, S2C and S2D), whereas it cannot form further assembly at neutral pH. DLS study supports the increase in the size of the assembly after siRNA complexation (Figure S4). Upon visible light irradiation, the spherical nanostructures of siRNA-HDA complex readily disassembled supporting the photo-responsiveness of the supramolecular assembly (Figure 3B).

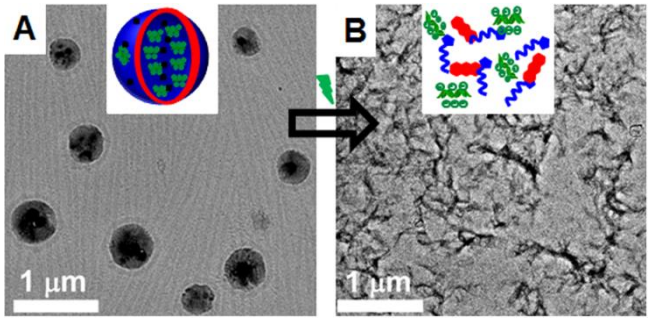


Figure 3. TEM images of (A) spherical siRNA-HDA assembly; (B) siRNA-HDA complex disassembly after green light (Black ray® B-100YP lamp, >500nm) irradiation for 40 min. in the presence of photosensitizer.

We then carried out fluorescence spectral titration to determine the interaction and binding constant between HDA and siRNA (Figure S5). Variation in fluorescence intensity of Cy3 (cyanine3) labeled siRNA in the presence of HDA was measured. In the absence of HDA, the fluorescence of Cy3siRNA showed no considerable change however upon addition of HDA, the fluorescence intensity at 564 nm decreased gradually (Figure S5A) and a growing new peak at 548 nm (Figure S5C) was seen as the concentration of HDA increased. The increase in the intensity of the peak at 548 nm could be due to the formation of a higher-order complex of cy3siRNA with HDA. The fluorescence of the complexing HDA with Cy3siRNA was correlated with a Job's plot that indicated a 1:1 (which equals 20 N/P ratio; N/P = No. of protonated amines from HDA/ No. of phosphate group from siRNA) stoichiometry (Figure S5D). The association constant for the complexation process was estimated to be 5.09 x 10^5 M^{-1} (error < 10%) following the modified Benesi–Hildebrand equation.[15] Gel electrophoresis was further employed to verify the ability of HDA to complex siRNA and then controllably release it upon exposure to green light (Figure S6). HDA complexed siRNA in acidic pH at N/P ratio 20 (Figure S6, lane 6) whereas it cannot complex siRNA in neutral pH at identical N/P ratio (Figure S6, Lane 4). The siRNA was released from HDA after green light exposure for 40 min in the presence of eosin (Figure S6, lane 7). The critical aggregation concentration of HDA in the presence of siRNA was measured by monitoring the dependence of the optical transmittance at 600 nm on the concentration of HDA. In the absence of siRNA, the optical transmittance of HDA at 600 nm showed no appreciable change as the concentration increased until 0.15 mM (Figure S5E, Supporting Information). However, at fixed HDA concentration (0.10 mM), the optical transmittance decreased gradually as the concentration of siRNA increased because of the formation of the large amphiphilic assembly. Figure S5 showed that the HDA/siRNA complex critical aggregation concentration was obtained as 4.5−7.5 μM (Figures S5F).

CCK-8 assay was used to assess the biocompatibility of HDA assembly. As shown in Figure S7 HDA did not exhibit significant toxicity at their optimal concentrations for transfection in HeLa cells. Approximately 95% of the cells survived at each concentration of HDA. Cellular uptake by HeLa cells and controlled disassembly of siRNA-HDA complex (Figure 4A–C and S8–9) was monitored by confocal microscopy. Figures 4B and S8 reveal the presence of blue fluorescence in HeLa cells treated with cy3 labeled siRNA-HDA complex, indicating the uptake of the assembly. Upon visible light irradiation (40 minutes), the blue fluorescence disappeared confirming the photo-degradation of HDA while the green fluorescence of cy3siRNA was monitored supporting the successful delivery to cells. As a control, cells were treated with naked cy3siRNA under the same conditions but no green fluorescence could be detected in cells (Figure S9).

To examine the gene silencing effect of siRNA-HDA complex, we analyzed the Bcl-2 mRNA expression level by quantitative real-time PCR (qRT-PCR). The siBcl2–HDA could remarkably enhance the gene-silencing efficacy, resulting in more than 90% of gene knockdown after visible–light exposure (Figure 4D). As negative controls, Hela cells were also transfected by HDA complex with scramble/nonspecific sequence (siNS-HDA), showing no down regulation of Bcl-2 mRNA level, supporting the specific gene regulation effect of Bcl-2 siRNA.

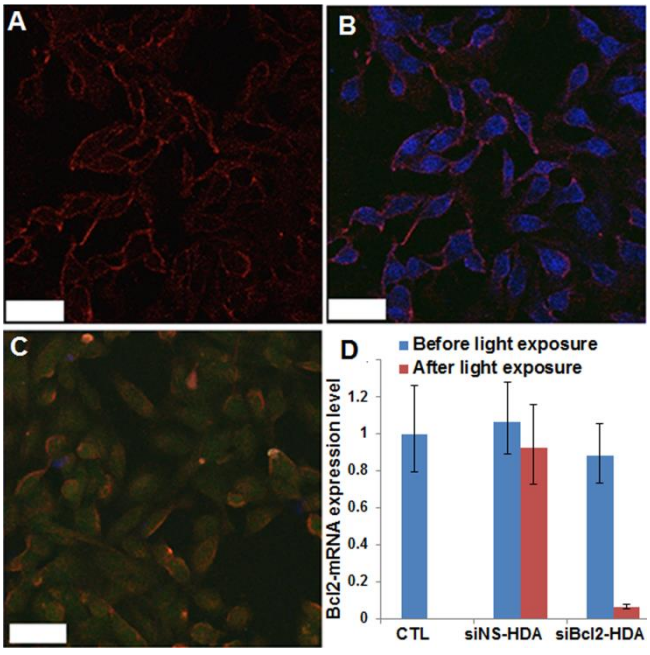


Figure 4. Confocal images of live HeLa cells after incubation with siRNA-HDA (A) Stained with plasma membrane dye; (B) Merged image before irradiation; (C) Merged image after 40 min. irradiation (scale bar 50 μM); (D) Expression level of Bcl-2 mRNA in Hela cells determined by qRTPCR; CTL: untreated cells; siNS–HDA: nonspecific–siRNA with HDA; siBcl2–HDA: Bcl-2 siRNA with HDA.

In summary, a visible light responsive supramolecular assembly was prepared employing histidine–capped–dialkoxy-anthracene building blocks. SCXRD revealed two types of hydrogen bonding involving the imidazole and amide moieties. When protonated in acidic aqueous solution, this assembly can successfully complex negatively charged siRNA and controllably release it upon visible light irradiation. This biocompatible platform could efficiently knock down Bcl-2 gene expression (up to 90%) in live cells on-demand. Thus, small molecule supramolecular self-assembly should be further investigated as a promising candidate for the preparation of single component siRNA transfecting agents.

Keywords: Self-assembly • Photo-responsive • Hydrogen bonding • Supramolecular • siRNA delivery

1) a) Y. Chang, K. Yang, P. Wei, S. Huang, Y. Pei, W. Zhao, Z. Pei, *Angew. Chem. Int. Ed.* **2014**, *53*, 13126; b) C. Wang, Z. Wang, X. Zhang, *Acc. Chem. Res.* **2012**, *45*, 608; c) S. Qin, Y. Geng, D. E. Discher, S. Yang, *Adv. Mater.* **2006**, *18*, 2905; d) K. T. Kim, J. J. L. M. Cornelissen, R. J. M. Nolte, J. C. M. van Hest, *Adv. Mater.* **2009**, *21*, 2787; e) X. Yan, D. Xu, X. Chi, J. Chen, S. Dong, X. Ding, Y. Yu, F. Huang, *Adv. Mater.* **2012**, *24*, 362; f) X. Ji, S. Dong, P. Wei, D. Xia, F. Huang, *Adv. Mater.* **2013**, *25*, 5725; g) K. Wang, C. -Y. Wang, Y. Wang, H. Li, C.-Y. Bao, J.-Y.; Liu, S. X.-A. Zhang, Y.-W. Yang, *Chem. Commun.* **2013**, *49*, 10528; h) S. Chen, Y. Ruan, J. D. Brown, J. Gallucci, V. Maslak, C. M. Hadad, J. D. Badjic´, *J. Am. Chem. Soc.* **2013**, *135*, 14964; i) G. Gasparini, E. K. Bang, J. Montenegro, S. Matile, *Chem. Commun.* **2015**, *51*, 10389; j) C. Gehin, J. Montenegro, E. K. Bang, A. Cajaraville, S. Takayama, H. Hirose, S. Futaki, S. Matile, H. Riezman, *J. Am. Chem. Soc.* **2013**, *135*, 9295.

2) a) T. Aida, E. W. Meijer, S. I. Stupp, *Science* **2012**, *335*, 813; b) X. Xu, H. Yuan, J. Chang, B. He, Z. Gu, *Angew. Chem. Int. Ed.* **2012**, *51*, 3130; c) J. L. Sessler, J. Jayawickramarajah, *Chem. Commun.* **2005**, 1939; d) L. C. Palmer, S. I. Stupp, *Acc. Chem. Res.* **2008**, *41*, 1674; e) A. R. Hirst, B. Escuder, J. F. Miravet, D. K. Smith, *Angew. Chem. Int. Ed.* **2008**, *47*, 8002.

3) a) A. Aydogan, J. L. Sessler, *Chem. Commun.* **2014**, *50*, 13600. (b) C. H. Ren, J. W. Zhang, M. S. Chen, Z. M. Yang, *Chem. Soc. Rev.* **2014**, *43*, 7257; b) J. W. Steed, *Chem. Commun.* **2011**, *47*, 1379; c) Y. Zhou, Y. Yao, M. Xue, *Chem. Commun.* **2014**, *50*, 8040; d) L. E. Buerkle, S. J. Rowan, *Chem. Soc. Rev.* **2012**, *41*, 6089; e) S. I. Stupp, *Nano Lett.* **2010**, *10*, 4783; f) J. A. Foster, J. W. Steed, *Angew. Chem. Int. Ed.* **2010**, *49*, 6718; g) Z. Yang, K. Xu, L. Wang, H. Gu, H. Wei, M. Zhang, B. Xu, *Chem. Commun.* **2005**, 4414; h) X. Li, K. Yi, J. Shi, Y. Gao, H.-C. Lin, B. Xu *J. Am. Chem. Soc.* **2011**, *133*, 17513; i) D.-S. Guo, Y. Liu, *Chem. Soc. Rev.* **2012**, *41*, 5907; j) K. Kim, N. Selvapalam, Y. H. Ko, K. M. Park, D. Kim, *J. Chem. Soc. Rev.* **2007**, *36*, 267; k) A. Harada, Y. Takashima, H. Yamaguchi, *Chem. Soc. Rev.* **2009**, *38*, 875.

4) a) N. M. Khashab, M. E. Belowich, A. Trabolsi, D. C. Friedman, C. Valente, Y. Lau, H. A. Khatib, J. I. Zink, J. F. Stoddart, *Chem. Commun. 2009*, 5371; b) L. Zhang, K. Yu, A. Einsenberg, *Science* **1996**, *272*, 1777; c) Y. Wang, N. Ma, Z. Wang, X. Zhang, *Angew. Chem. Int. Ed.* **2007**, *46*, 2823; d) S. Yagai, A. Kitamura, *Chem. Soc. Rev.* **2008**, *37*, 1520; e) X. Zhang, C. Wang, *Chem. Soc. Rev.* **2011**, *40*, 94; f) A. Klaikherd, C. Nagamani, S. Thayumanavan, *J. Am. Chem. Soc.* **2009**, *131*, 4830; g) Y. Yao, M. Xue, J. Chen, M. Zhang, F. Huang, *J. Am. Chem. Soc.* **2012**, *134*, 8711; h) G. Yu, M. Xue, Z. Zhang, J. Li, C. Han, F. Huang, *J. Am. Chem. Soc.* **2012**, *134*, 13248; i) J. Hu, G. Zhang, S. Liu, *Chem. Soc. Rev.* **2012**, *41*, 5933; j) S. Li, B. A. Moosa, J. G. Croissant, N. M. Khashab, *Angew. Chem. Int. Ed.* **2015**, *54*, 6804.

5) a) A. Altieri, G. Bottari, F. Dehez, D. A. Leigh, J. K. Y. Wong, F. Zerbetto, *Angew. Chem. Int. Ed.* **2003**, *42*, 2296; b) S. J. Vella, J. Tiburcio,S. J. Loeb, *Chem. Commun.* **2007**, 4752; c) H. Meier, *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1399; d) J.-F. Xu, Y.-Z. Chen, D. Wu, L.-Z. Wu, C.-H. Tung, Q.-Z. Yang, *Angew. Chem. Int. Ed.* **2013**, *52*, 9738.

6) a) D. Wang, G. Ye, Y. Zhu, X. Wang, *Macromolecules* **2009**, *42*, 2651; b) J. Barrio, L. Oriol, C. Sanchez, J. L. Serrano, A. D. Cicco, P. Keller, M. H. Li, *J. Am. Chem. Soc.* **2010**, *132*, 3762; c) B. Yan, J. C. Boyer, D. Habault, N. R. Branda, Y. Zhao, *J. Am. Chem. Soc.* **2012**, *134*, 16558.

7) a) M. Y. Jiang, D. Dolphin, *J. Am. Chem. Soc.* **2008**, *130*, 4236; b) D. Arian, L. Kovbasyuk, A. Mokhir, *J. Am. Chem. Soc.* **2011**, *133*, 3972; c) A. Meyer, A. Mokhir, *Angew. Chem. Int. Ed.* **2014**, *53*, 12840; d) M. Zamadar, G. Ghosh, A. Mahendran, M. Minnis, B. I. Kruft, A. Ghogare, D. Aebisher, A. Greer, *J. Am. Chem. Soc.* **2011**, *133*, 7882.

8) N. Idris, M. K. Gnanasammandhan, J. Zhang, P. C. Ho, R. Mahendran, Y. Zhang, *Nature Med.* **2012**, *18*, 1580.

9) a) Q. Yan, J. Hu, R. Zhou, Y. Ju, Y. Yina, J. Yuan, *Chem. Commun.* **2012**, *48*, 1913; b) S. Yang, N. J. Li, Z. Liu, W. Sha, D. Chen, Q. Xu, J. Lu, *Nanoscale* **2014**, *6*, 14903; (c) S. Yang, N. Li, D. Chen, X. Qi, Y. Xu, Y. Xu,Q. Xu, H. Li, J. Lu, *J. Mater. Chem. B*, **2013**, *1*, 4628.

10) Y. X. Wang, Y. M. Zhang, Y. Liu, *J. Am. Chem. Soc.* **2015**, *137*, 4543.

11) Y. W. Cho, J. D. Kim, K. J. Park, *Pharm. Pharmacol.* **2003**, *55*, 721; b) K.-L. Chang, Y. Higuchi, S. Kawakami, F. Yamashita, M. Hashida, *Bioconjugate Chem.* **2010**, *21*, 1087.

12) A. Kathryn, K. A. Whitehead, R. Langer, D. G. Anderson, *Nat. Rev. Drug Discov.* **2009**, *11*, 129.

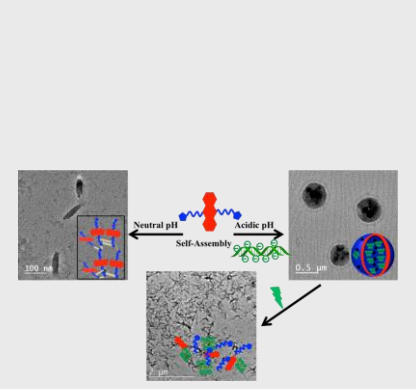
13) a) K. Raemdonck, B. Naeya, K. Buyens, R. E. Vandenbroucke, A. Hogset, J. Demeester, S. C. De Smedt, *Adv. Funct. Mater.* **2009**, *19*, 1406; b) R. Kanasty, J. R. Dorkin, A. Vegas, D. Anderson, *Nat. Mater.* **2013**, *12*, 967; c) L. Cui, J. L. Cohen, C. K. Chu, P. R. Wich, P. H. Kierstead, J. M. Frechet, *J. Am. Chem. Soc.* **2012**, *134*, 15840; d) B. J. Hong, A. J. Chipre, S. T. Nguyen, *J. Am. Chem. Soc.* **2013**, *135*, 17655.

14) a) H. Lee, A. K. R. Lytton-Jean, Y. Chen, K. T. Love, A. I. Park, E. D. Karagiannis, A. Sehgal, W. Querbes, C. S. Zurenko, M. Jayaraman, C. G. Peng, K. Charisse, A. Borodovsky, M. Manoharan, J. S. Donahoe, J. Truelove, M. Nahrendorf, R. Langer, D. G. Anderson, *Nat. Nanotechnol.* **2012**, *7*, 389.

15) I. J. Lee, S. P. Patil, K. Fhayli, S. Alsaiari, N. M. Khashab, *Chem. Commun.* **2015**, *51*, 3747.

COMMUNICATION

A "Smart Assembly": Visible light responsive small molecule self-assembly was developed employing histidine-capped-9,10-dialkoxy-anthracene. When protonated, this biocompatible platform could successfully complex siRNA and deliver it with high efficacy, upon visible light irradiation, leading up to 90% of gene silencing in live cells.



*Sachin P. Patil, Basem A. Moosa, Shahad Alsaiari, Kholod Alamoudi, Karim Adil, Mohamed Eddaoudi, and Niveen M. Khashab **

Page No. – Page No.

Supramolecular Self-assembly of Histidine-Capped-Dialkoxy-Anthracene: A Visible Light Triggered Platform for facile siRNA Delivery