ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 2071–2073

www.rsc.org/chemcomm

COMMUNICATION

Immobilization-free screening of aptamers assisted by graphene oxide†

Jee-Woong Park,‡[a] Rameshwar Tatavarty,‡[a] Dae Woo Kim,[b] Hee-Tae Jung[b] and Man Bock Gu*[a]

Received 18th October 2011, Accepted 23rd November 2011
DOI: 10.1039/c2cc16473f

Graphene oxide (GO) has the ability to separate free short ssDNA in heterogeneous solution. This feature is applied as a label free platform for screening of aptamers that bind to their target with high affinity and specificity. Herein, we report an aptamer selection strategy for Nampt protein based on GO.

It has been proven that ssDNA can strongly interact *via* π–π stacking and get adsorbed on two dimensional graphene and graphene oxide (GO) sheets.[1] The phenomenon of GO adsorbing DNA and its interaction with proteins and small molecules has been explored as assays and a sensing platform.[2] The dsDNA adsorption on GO is very weak due to the shielding of nucleobases by the phosphate backbone, whereas ssDNA binds much strongly on GO.[3] This indifference of ssDNA/dsDNA and graphene is exploited to assay nucleic acid unwinding activity of helicases.[2g] The kinetics of adsorption and desorption of ssDNA on GO is well discussed and reported in detail.[1] In brief, shorter ssDNA shows better adsorption. The desorption of ssDNAs from GO is only possible either by hybridization with a complementary sequence or in very few cases (*e.g.*, aptamers) conformational change induced by target protein or small molecules.[1,4]

Aptamers are short ssDNA/RNA sequences which bind with high specificity and affinity to the targets including proteins and small molecules. Systematic evolution of ligands by exponential enrichment (SELEX) is a process to isolate these nucleic acid sequences, which functionally bind and discriminate their target in a complex heterogeneous environment.[5] A typical SELEX technique involves three steps: binding, separation, and amplification, a number of variations in the technique have been incorporated resulting in aptamer generation with high affinity and specificity.[5a,b,6] Techniques have been designed to improve a previous methodology and necessitated specialized steps or instruments for target immobilization or specific experimental setup (*e.g.* Capillary electrophoresis, Atomic force microscopy, Microfluidics, Sol–gel array).[6b–e,7] However, most SELEX protocols are still inherently tedious and complex. This desire to succeed SELEX without immobilization of unlimited targets still remains. This has led to the development of immobilization free SELEX based on GO. Herein, we report a simple, easy and very efficient technique based on GO to derive aptamers without target immobilization. The non-specific adsorption of ssDNA random library on GO is the basis of separation of unbound DNA in the GO-SELEX process.

In our study, we chose one of the adipokines 'Nampt' as a model protein, since it's blood plasma levels are attributed to obesity and obese related metabolic diseases such as Type 2 diabetes, mellitus or cardiovascular diseases.[8]

As a proof of concept, we report herein a novel and versatile GO-SELEX process for the identification of enriched aptamers for Nampt based on the affinity of ssDNA to GO. The GO-SELEX procedure reduces a number of steps involved in the conventional SELEX process.

The preliminary experiments were performed to find the adsorption ratio of ssDNA library on synthesized GO by previously reported procedures.[9] The adsorption ratio of 70 bp ssDNA/GO was found to be 10 μg mg^{-1} of GO. In another experiment, two thrombin binding aptamers with extensive G-quadruplex structure (15 bp and 29 bp) show complete adsorption at 50 pmoles mg^{-1} of GO (working conc. of ssDNA library in SELEX).[10]

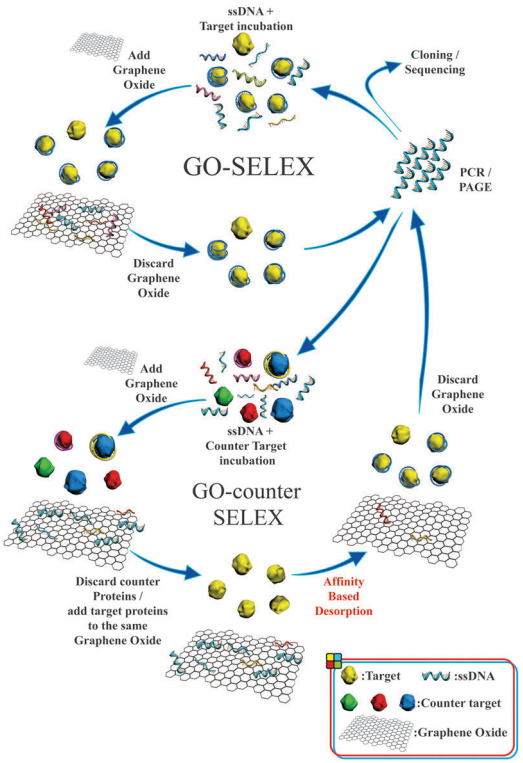
It should be noted that it is impossible to amplify the ssDNA library at any stage when the target is not added in the GO-SELEX. The maximum wt. ratio of ssDNA : GO at any stage was kept at 1 : 1000. To the best of our knowledge, we are not aware of any short ssDNA sequence which does not get adsorbed on GO including the well known ssDNA that possesses G-quadruplex structure.[10] In brief, all short ssDNA are adsorbed and get separated from the solution unless bound to protein.

The individual steps involved in one GO-SELEX and GO-counter SELEX round are illustrated in Scheme 1. In the first step of affinity selection, the target protein (Nampt) was mixed with the ssDNA random library. This step would allow a certain population of DNA sequences from the ssDNA pool to bind to the target protein freely in solution. Subsequently, the mixture of potential aptamers bound to target and unbound ssDNA was incubated with GO solution. The free unbound ssDNAs in the solution get adsorbed on GO

[a] *College of Life Sciences and Biotechnology, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-713, South Korea. E-mail: mbgu@korea.ac.kr; Fax: +822-928-6050; Tel: +822-3290-3417*
[b] *Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science & Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, South Korea. Fax: +82 42-350-8890; Tel: +82 42-350-3931*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2cc16473f

‡ These authors contributed equally to this work.



Scheme 1 Schematic illustration of the steps involved in the GO-SELEX procedure of aptamer identification. In the first separation step of the GO-SELEX process, a preincubated mixture of random ssDNA library and target is added to GO; the unbound ssDNA gets adsorbed on GO and is discarded. The target bound ssDNA are recovered, amplified and purified for further GO-counter SELEX process. In the GO-counter SELEX process, the recovered ssDNA is incubated with counter targets and mixed with GO, the counter target bound ssDNA is discarded and the ssDNA bound to GO that are the potential aptamers are recovered by affinity based desorption through addition of target to ssDNA bound GO, the recovered ssDNA is thus purified, cloned and sequenced to obtain the final hit aptamers.

via π–π stacking, while ssDNAs bound to target remain in solution. The pH of the Tris-HCl (7.4) is kept at neutral or closer to the isoelectric point (PI) of the Nampt (PI = 7.0) to minimize polar and charged interaction between protein and GO which is negatively charged due to the presence of the carboxylic acid and phenolic hydroxyl groups.[11] In the separation step, the ssDNA bound GO was removed by centrifugation and the supernatant with ssDNAs bound to the target was purified and recovered by ethanol precipitation. All the steps in one round of GO-SELEX can be performed in 240 min, whereas GO-counter SELEX requires 390 min. The recovered ssDNA library was amplified by PCR and ssDNA from the dsDNA PCR product were separated by PAGE. With the purified library, subsequent rounds of GO-SELEX were performed. To eliminate false positive binding of aptamers, the counter-SELEX step is employed in which the focused ssDNA library is screened against the closely related structures and targets that are more likely to be present in a complex heterogeneous environment. To incorporate this counter-SELEX step with GO-SELEX, we relied on the affinity based desorption of ssDNAs from the GO.

Once the saturation in recovery ratio of ssDNAs was observed in SELEX rounds, the GO counter-SELEX procedure was initiated. In the GO-counter SELEX, a mixture of potential counter targets is mixed with a ssDNA pool from the previous SELEX round. Any ssDNAs adsorbed on GO at this stage are likely to be specific aptamers for the target used in previous SELEX rounds. For the recovery of the adsorbed ssDNA on GO, affinity based desorption was triggered by incubating the GO with the target. During affinity based desorption, when the target gets in contact with potential aptamers immobilized on GO, the target interaction with its cognate aptamer induces conformational change in the ssDNA aptamer refolding itself in specific conformation for binding to the target, that in turn weakens the π–π stacking interactions with the GO surface, causing its release from GO. The aptamers obtained *via* desorption are extracted, amplified and progressed to the final SELEX round.

Initially, 200 pmoles of random library and Nampt were incubated with 1 : 1 molar ratio. After separation of unbound ssDNA with GO, the ssDNA bound to Nampt were amplified and purified by PCR and PAGE separation and subjected to successive rounds of affinity selection. At each round the recovered ssDNA was quantified by UV visible spectroscopy. As expected, we observed a linear increase in the recovery ratio of ssDNA bound to Nampt from round 1 to round 3, after which there was no increase in recovery ratios for two consecutive (4th and 5th) GO-SELEX rounds, indicating that the DNA sequence population in the random library was enriched with sequences specific to Nampt (Fig. 1). When the saturation in recovery of ssDNA was observed, the GO counter-SELEX was performed in the following round. For the GO counter-SELEX, Human serum albumin (HSA) and four other adipokines: Retinol binding protein 4 (RBP4), Adiponectin, Resistin, and Vaspin were chosen. Since in the GO-SELEX procedure we used a 1 : 1 molar ratio of ssDNA library to Nampt to ensure that each ssDNA molecule interacts with a single molecule of target, similarly in the GO-counter

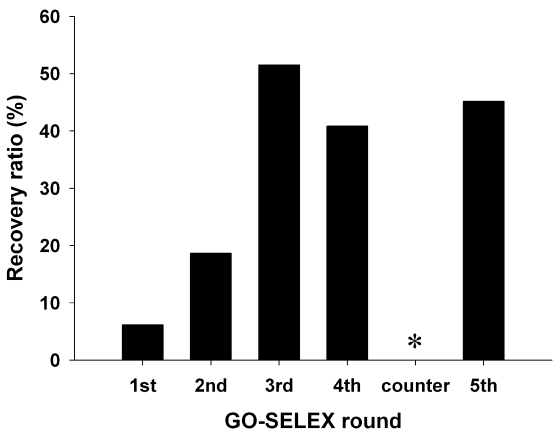


Fig. 1 Recovery ratio of target bound ssDNA library pool after ethanol precipitation. The graph shows that GO efficiently traps the unbound ssDNA and the ratio of recovered target bound ssDNA after amplification increases with each GO-SELEX round, with saturation in recovery observed after the 4th round of GO-SELEX. The (*) represents the GO-counter SELEX round performed after the 4th round of GO-SELEX respectively.

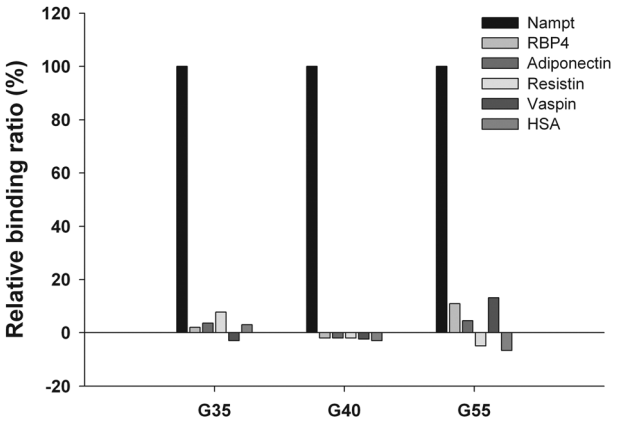


Fig. 2 The specificity of the selected aptamers G35, G40, G55 for Nampt by surface plasmon resonance assay. The specificity of the aptamer sequences was analyzed by immobilizing each of the individual 15 ssDNA sequences obtained after cloning on an SPR gold chip. The SPR analysis shows that G35, G40, G55 showed specific binding only to Nampt with extremely low or no binding to four adipokines (RBP4, Adiponectin, Resistin, and Vaspin) and human serum albumin (HSA).

SELEX, the amount of each counter target protein was 50 pmoles and all five counter targets were incubated with the ssDNA library pool and then on GO. The aptamers bound to GO were recovered by affinity based desorption by incubation with 200 pmoles of Nampt (ESI†). The recovered ssDNA was subjected to the fifth round of SELEX. After GO-counter SELEX and a subsequent round of SELEX, the recovery ratio was found to be similar to the previous (4th) round of GO-SELEX. The data demonstrate that the GO-SELEX process could efficiently eliminate the unbound ssDNA sequences and the affinity desorption based recovery of ssDNA from GO-counter SELEX resulted in an enriched population of ssDNA aptamer sequences specific for the chosen Nampt. The enriched ssDNAs were cloned and sequenced. Finally, 48 sequences were derived and they were ranked by Gibb's free energy (Table S1 in ESI†). To further test the specificity of the obtained hit aptamer sequences, we utilized surface plasmon resonance (SPR) based assay for their affinities and specificities against four other adipokines and HSA. We identified three aptamer sequences that showed very high specificity for Nampt (Fig. 2, ESI†). To evaluate the binding affinity of the identified aptamers a dose dependent assay was performed for all three aptamers to obtain their dissociation constant, K_d values (Fig. 3). The calculated K_d values for aptamers G35 and G40 were found to be 117.4 nM and 114.4 nM, respectively, while aptamer G55 showed the lowest K_d value of 71.7 nM.

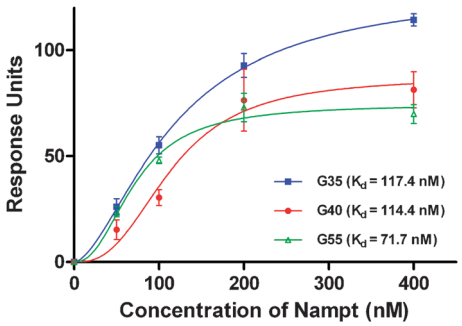


Fig. 3 The dose dependent saturation plots for Nampt binding to aptamer sequences G35, G40 and G55 obtained by SPR assay.

In conclusion, by exploiting the ability of GO to adsorb ssDNAs a novel, simple to use, cost effective, immobilization-free new SELEX method is demonstrated *in vitro*. The target induced conformational change of aptamers initiates the affinity based desorption of ssDNAs from the GO. The GO-SELEX required less than five SELEX rounds to obtain aptamers with high affinity and specificity. More importantly a major advantage of the GO-SELEX methodology is that it requires reduced amounts of targets. To the best of our knowledge, we have implemented for the first time the most cost effective, simplified, rapid and immobilization-free SELEX without the need for specialized instruments to obtain aptamers.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2010-00579-KORANET) and Technical Transfer funded by the Small & Medium Business Administration (SMBA) (Project #S1071611).

Notes and references

1 M. Wu, R. Kempaiah, P.-J. J. Huang, V. Maheshwari and J. Liu, *Langmuir*, 2011, **27**, 2731.
2 (*a*) H. X. Chang, L. H. Tang, Y. Wang, J. H. Jiang and J. H. Li, *Anal. Chem.*, 2010, **82**, 2341; (*b*) B. Li, S. Dong and E. Wang, *Chem.–Asian J.*, 2010, **5**, 1262; (*c*) M. Zhou and S. Dong, *Acc. Chem. Res.*, 2011, **44**, 1232; (*d*) M. Zhou, Y. M. Zhai and S. J. Dong, *Anal. Chem.*, 2009, **81**, 5603; (*e*) C.-H. Lu, H.-H. Yang, C.-L. Zhu, X. Chen and G.-N. Chen, *Angew. Chem., Int. Ed.*, 2009, **48**, 4785; (*f*) A. Bonanni and M. Pumera, *ACS Nano*, 2011, **5**, 2356; (*g*) H. Jang, Y. K. Kim, H. M. Kwon, W. S. Yeo, D. E. Kim and D. H. Min, *Angew. Chem., Int. Ed.*, 2010, **49**, 5703; (*h*) J. H. Jung, D. S. Cheon, F. Liu, K. B. Lee and T. S. Seo, *Angew. Chem., Int. Ed.*, 2010, **49**, 5708; (*i*) Y. M. Lin, A. Valdes-Garcia, S. J. Han, D. B. Farmer, I. Meric, Y. N. Sun, Y. Q. Wu, C. Dimitrakopoulos, A. Grill, P. Avouris and K. A. Jenkins, *Science*, 2011, **332**, 1294.
3 S. J. He, B. Song, D. Li, C. F. Zhu, W. P. Qi, Y. Q. Wen, L. H. Wang, S. P. Song, H. P. Fang and C. H. Fan, *Adv. Funct. Mater.*, 2010, **20**, 453.
4 C.-H. Lu, J. Li, M.-H. Lin, Y.-W. Wang, H.-H. Yang, X. Chen and G.-N. Chen, *Angew. Chem., Int. Ed.*, 2010, **49**, 8454.
5 (*a*) C. Tuerk and L. Gold, *Science*, 1990, **249**, 505; (*b*) A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818; (*c*) M. X. You, Y. Chen, L. Peng, D. Han, B. C. Yin, B. C. Ye and W. H. Tan, *Chem. Sci.*, 2011, **2**, 1003.
6 (*a*) R. Stoltenburg, C. Reinemann and B. Strehlitz, *Anal. Bioanal. Chem.*, 2005, **383**, 83; (*b*) S. D. Mendonsa and M. T. Bowser, *Anal. Chem.*, 2004, **76**, 5387; (*c*) X. Lou, J. Qian, Y. Xiao, L. Viel, A. E. Gerdon, E. T. Lagally, P. Atzberger, T. M. Tarasow, A. J. Heeger and H. T. Soh, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 2989; (*d*) Y. Miyachi, N. Shimizu, C. Ogino and A. Kondo, *Nucleic Acids Res.*, 2009, **38**, e21; (*e*) S. M. Park, J. Y. Ahn, M. Jo, D. K. Lee, J. T. Lis, H. G. Craighead and S. Kim, *Lab Chip*, 2009, **9**, 1206.
7 S. S. Oh, J. Qian, X. Lou, Y. Zhang, Y. Xiao and H. T. Soh, *Anal. Chem.*, 2011, **83**, 1866.
8 (*a*) T. D. Filippatos, H. S. Randeva, C. S. Derdemezis, M. S. Elisaf and D. P. Mikhailidis, *Curr. Vasc. Pharmacol.*, 2010, **8**, 12; (*b*) D. G. Haider, K. Schindler, G. Schaller, G. Prager, M. Wolzt and B. Ludvik, *J. Clin. Endocrinol. Metab.*, 2006, **91**, 1578.
9 (*a*) B. S. Kong, J. X. Geng and H. T. Jung, *Chem. Commun.*, 2009, 2174; (*b*) W. S. Hummers and R. E. Offeman, *J. Am. Chem. Soc.*, 1958, **80**, 1339.
10 D. M. Tasset, M. F. Kubik and W. Steiner, *J. Mol. Biol.*, 1997, **272**, 688.
11 D. Li, M. B. Muller, S. Gilje, R. B. Kaner and G. G. Wallace, *Nat. Nanotechnol.*, 2008, **3**, 101.