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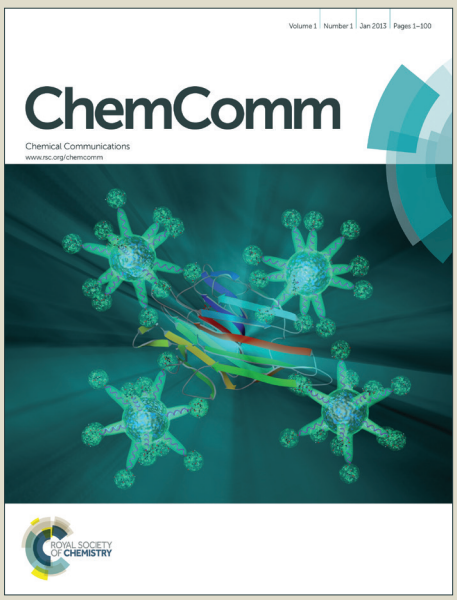

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Strain-Promoted Alkyne- Azide Cycloaddition (SPAAC) reaction of novel EpCAM aptamer-fluorescent conjugate for cancer cells imaging

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For the first time, a novel EpCAM aptamer (SYL3C)-DIBO-AF594 fluorescent conjugate was synthesised by bioorthogonal chemistry utilizing strain promoted alkyne-azide cycloaddition (copper free click) reaction (SPAAC). The ligation efficiency of SPAAC was improved by freeze-thaw cycle. The obtained conjugate showed target specific binding and aided in imaging of various EpCAM positive cancer cell lines like MCF7, MDAMB453, Weri-RB1 and PC3.

Cancer is a major cause of death worldwide. Moreover, millions of people are diagnosed with some type of cancer every year. Since early detection may aid in disease prevention, there is considerable interest around the world for cancer diagnosis. Cancer diagnosis by molecular-imaging is gaining importance due to identification of many cancer cell specific markers.[1] Cancer cell specific non-invasive imaging by the use of molecular probes such as aptamers,[2,3] antibodies, affibodies, small molecules, peptides[4] and nanocarriers[5] are currently being explored.

Epithelial cell adhesion molecule (EpCAM) is a multifunctional molecule present on the cell surface, mediating cell signalling.[6] It is over expressed in epithelial cancers as well in retinoblastoma, leading to its exploration in molecular imaging and targeted therapy. Hence EpCAM is used as diagnostic marker, [7] potential target for immunotherapeutic strategies,[8] and for delivery of therapeutic antibodies, affibodies and ankyrins.[9-11]

Aptamers are single strands of DNA or RNA produced from a process termed "systematic evolution of ligands by exponential enrichment" (SELEX).[12] These aptamers can be conjugated with fluorescent or radio labels for studies related to diagnostic and therapeutic aspects of cancer respectively.[13] Owing to their stability, less immunogenicity, less toxicity, ease of synthesis and handling, aptamers has emerged as a promising candidates for theranostic purpose over protein or peptide drugs.[14,15] EpCAM RNA aptamer based delivery of doxorubicin to retinoblastoma cells sparing the normal cells was earlier shown by our group.[16] Duan W *et al.*, described EpCAM aptamer functionalized PLGA-lecithin-curcumin-PEG nanoparticles targeting colorectal adenocarcinoma cells.[17] Moreover, EpCAM aptamers are involved in various cancer targeting.[18]

Carrying out synthetic manipulations under biological environment without interfering with the concurrent biological processes is a challenging task. Bioorthogonal chemistry was developed specifically to address this.[19] One such protocol having profound importance is strain promoted alkyne - azide cylcoaddition (copper free click) reaction (SPAAC).[20,21] This reaction had received considerable attention owing to the simplicity and faster reaction rates.

SPAAC had been utilized for ligation between two fragments of a DNA split aptamer mediated by a small molecule cocaine. Those ligated complexes were used for sensing purposes.[22] In order to achieve site-specific introduction of the requisite probes, Delft *et al*., described terminal modification of oligonucleotide with bicyclo[6.1.0]nonyne (BCN) or azide. However, the synthetic application of the probes for specific targeting of cancers was not reported.[23]

Song *et al.,* developed DNA aptamer against EpCAM (EpDNA) and utilized it for detecting EpCAM overexpressing cancer cells from normal cells.[24] The aptamer biocompatibility was assayed by cancer cell capturing from the different cell populations. However, aptamer conjugate synthesis using bioorthogonal reactions were not reported.

Although various reports described either EpCAM aptamer for cancer targeting or SPAAC for bioorthogonal ligation, we were intrigued by the lack of aptamer based bioorthogonal reactions targeting EpCAM positive cancers. It will be of paramount

importance to develop bioorthogonal approaches that will expand the utility of the aptamer conjugates. This in turn will exponentially open up the synthetic schemes available to researchers. Hence it was decided to develop bioorthogonal reactions for the aptamer conjugation using SPAAC for EpCAM cancer cell specific imaging.

In the present study, we utilized the fluorescent tagged alkyne, (alkyne-DIBO) to generate novel EpCAM aptamer conjugate for cancer cell imaging. The aptamer sequence (SYL3C) used in the current work has been reported for its EpCAM specificity.[24] This obviated the need to use a scrambled aptamer to prove the sequence specificity. Hence no random sequence was included in the present work. Instead, EpCAM low expressing cell line was used as control. Alexa-flour 594 (AF594) fluorescent dye conjugated DIBO was used for the cycloaddition with azide-end terminated aptamer to generate conjugate (EpDNA-DIBO-AF594) for fluorescent imaging (Figure 1).

The SPAAC reaction scheme for the conjugation of aptamer carrying terminal azide modification with an alkyne, DIBO is presented in Figure 2A. Initially, a set of experiments were performed by varying the equivalent ratio of aptamer to DIBO-AF594 (1:1, 1:2, 1:4, 1:6) at room temperature in 10 nM HEPES (pH 7.0) (Gibco, Invitrogen bioscience, Bangalore, India) buffer.

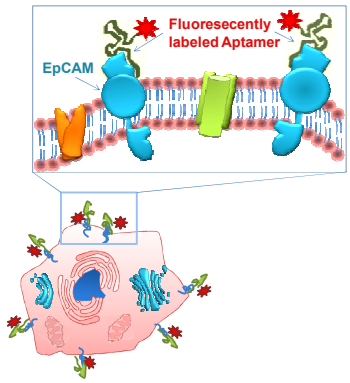

Figure 1. Illustration showing the imaging of cancer cell by targeting EpCAM on the cell surface using fluorescent EpCAM aptamer conjugate generated by strain promoted alkyne-azide cycloaddition reaction.

However, it was observed that the change of ratios did not increase either the conversion or the yield in a significant way. At higher ratios of DIBO, we observed the purification of unreacted DIBO becoming cumbersome. Hence 1:1 ratio was used for further studies (Figure 2B).

It was reported that freeze thaw can be effectively utilized to increase the ligation efficiency.[25] In the present study also it was observed that repeated freeze (-30°C) and thaw (4°C) increased the EpDNA-DIBO-AF594 formation (conversion from 35% to 75% HPLC analysis) (Figure S1).

The retention time corresponding to HPLC traces at 27.33 and 27.81 min were presumed to be regioisomers of the EpDNA-DIBO-AF594 (same mass by MALDI) and hence no attempts were made to separate them. The final purification of the EpDNA-DIBO-AF594 was carried out by collecting, pooling, concentrating and desalting the fractions corresponding to the retention time 27.33 and 27.81 min. Mass spectrum (MALDI-TOF) of the purified EpDNA-DIBO-AF594 is depicted in Figure 3.

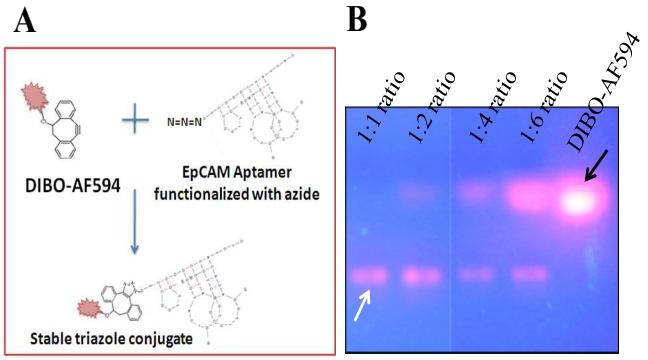

Figure 2. A. Scheme illustrating reaction of azide terminated EpCAM with DIBO-AF594 to generate fluorescent aptamer conjugate by strain promoted alkyne-azide cycloaddition. B. Gel electrophoresis of unpurified Aptamer-DIBO reaction with increasing concentration of DIBO.

As a control experiment, it was decided to study the stability of the EpDNA-DIBO-AF594 in PBS at physiological temperature and pH. The EpDNA-DIBO-AF594 was intact up to 72 hours (agarose gel electrophoresis) (Figure S2A). Also dialysis did not show any degradation or release of DIBO-AF594 or AF594 (Figure S2B).

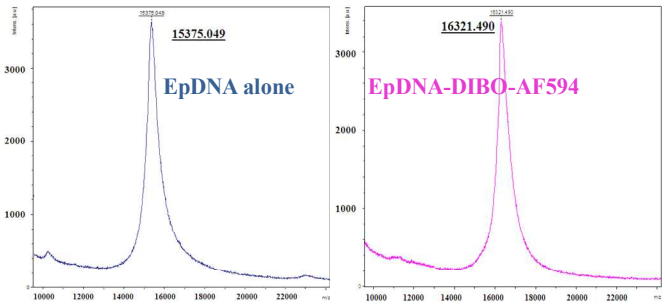

Figure 3. MALDI-ToF analysis of the aptamer alone (left panel) and EpDNA-DIBO-AF594 purified from HPLC (right panel) (mass observed were underlined)

After successfully synthesizing and purifying the aptamer-fluorescent conjugate, focus was shifted to the binding and internalization studies on EpCAM positive cancer cell lines. Since the EpCAM is differently expressed in different cancer cell lines, particular importance was given to study the binding and internalization of the EpDNA-DIBO-AF594 in the following cell lines. Breast cancer cell line: MCF7, MDAMB453, retinoblastoma cell line: Weri-Rb1, prostate cancer cell line: PC3 and low EpCAM expressing -Muller glial cell line: MIO-M1. We had studied percentage EpCAM expression in cell lines and found that MCF7 and MDAMB453 cell lines express EpCAM over 80 to 90% followed by PC3 and Weri-RB1 with 35 to 45%. The EpCAM expression in MIO-M1 cells was very less with 5% (data not shown). In order to differentiate between the membrane bound aptamer and the

internalized ones, incubation was carried out at two different temperatures and were analyzed either by flow cytometry or fluorescent microscopy. Incubation at 4°C selectively inhibits active cellular uptake hence membrane bound aptamer could be determined (flow cytometry). Incubation at 37°C allows the aptamer to internalize hence it can be visualized (fluorescent microscopy).[26]

The mean fluorescence intensity (MFI) of the total populations is represented in the Table 1. It became evident from the table that the EpDNA-DIBO-AF594 bound cells showed higher MFI in all EpCAM expressing cells in line with their EpCAM expression levels. The EpDNA-DIBO-AF594 bound MDAMB453 and PC3 cells showed significantly higher MFI compared to DIBO-AF594 bound cells MFI. Although MIO-M1 control cell line was used for the study, the DIBO showed nonspecific binding (data not shown).

	Mean Fluorescence Intensity (Total population)			
Cell lines	**MDAMB453**	**MCF7**	**Weri-Rb1**	**PC3**
Control	6.7	6.8	3	5.02
DIBO alone	8.23	6.83	4.52	8.11
EpDNA-DIBO-AF594	33.4	40.25	28.54	13.05

Table 1. Flow cytometry analysis of the binding of the EpDNA-DIBO-AF594 onto cell lines. The averaged Mean fluorescence intensities from triplicate experiments were represented.

The internalization of the EpDNA-DIBO-AF594 was visualized using inverted fluorescent microscope (Carl Zeiss, Germany) as well by dark field imaging using CytoViva microscope (Auburn, AL).[26] CytoViva imaging under dark field and fluorescence channels was performed to visualize the presence of aptamer within the cells by Z-stacking. Z-stacking from uppermost to lowermost cell surface clearly showed the presence of the fluorescence signal from the middle sections, indicating the internalization of aptamer EpDNA-DIBO-AF594. Cytoplasmic and nuclear staining was observed, due to the internalized EpDNA-DIBO-AF594 in MCF7, MDAMB453, Weri-Rb1, PC3 cells. Increased uptake of aptamer was observed in MCF7 & MDAMB453 followed by Weri-Rb1, while moderate intensity of fluorescence relating to lower aptamer accumulation was observed in the case of PC3 (Figure 4). The fluorescent signal from the internalized aptamer present in the cell cytoplasm and nuclear compartment is indicated by the white arrows. Fluorescent microscopy images showed similar internalization pattern for the cell lines mentioned above and no internalization was observed in MIO-M1 cells (Figure S3).

The binding of aptamer EpDNA-DIBO-AF594 studied by flow cytometry and microscopy in MCF7, MDAMB453, Weri-Rb1, PC3 and MIO-M1 cells were in line with similar pattern of EpCAM protein expression in the corresponding cell lines. As DIBO alone was reported for its binding on to fixed cells,[#] live cells without fixing were used in the study. Moreover, to minimize the non-specific binding of DIBO-AF594, a 2% FBS was used during the binding and internalization studies.

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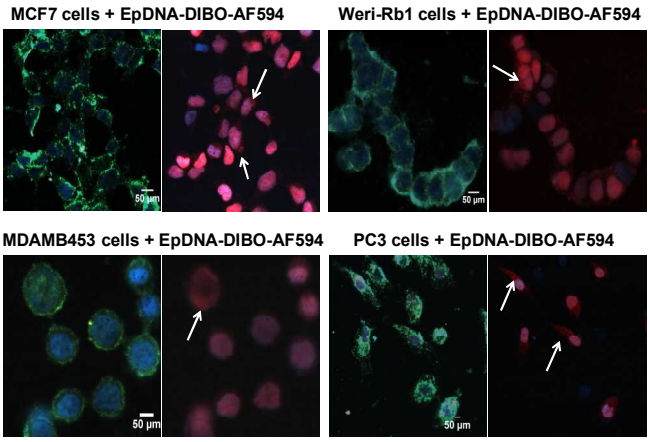

Figure 4. Dark field -Fluorescent microscopic imaging of EpDNA-DIBO-AF594 in cancer cells. Left panel of each cell line corresponds to: Dark field+DAPI and on the right panel: DAPI+AF594 merged. Scale bar indicates 50µm.

The cytotoxicity of EpDNA-DIBO-AF594 was carried out in MCF7 (high EpCAM expression), Weri-RB1 (moderate EpCAM expression) and MIO-M1 (low EpCAM expression) cells. MTT assay was performed after 48 hours of treatment. The EpDNA-DIBO-AF594 was tested from 100 nM to 1 µM concentration. Significant toxicity was not observed until 1 µM of EpDNA-DIBO-AF594 (Figure 5).

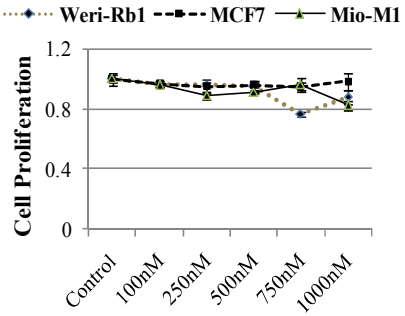

Figure 5. Effect of EpDNA-DIBO-AF594 on cell proliferation. The cytotoxicity of the EpDNA-DIBO-AF594 was evaluated by MTT assay after 48hrs of addition to cells. Control cells without treatment was normalized to 1. Experiments were performed in triplicates.

The EpDNA-DIBO-AF594 can be used up to 1µM concentration for imaging studies without compromising either cell proliferation or cellular metabolism. Although serum stability studies of the EpDNA-DIBO-AF594 up to 24 hr showed no significant degradation, there is 70% of degradation after 36 hr. Hence for therapeutic application that requires longer stay of the EpDNA-DIBO-AF594 in physiological environment, suitable modifications may be required (2'OMe or LNA or 3' idT). Thus EpDNA-DIBO-AF594 was successfully synthesized using bioorthogonal chemistry. It was also shown that the EpDNA-DIBO-AF594 can be successfully used to image EpCAM positive cancer cell lines. This novel concept can be potentially applied for similar *in vivo* studies and can be used for imaging of various cancers. Further work to

incorporate radiometals to aid in PET imaging and for therapeutic purpose is currently underway.

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Notes and references

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