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Aptamers against Cells Overexpressing Glypican 3 from Expanded Genetic Systems Combined with Cell Engineering and Laboratory Evolution

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Abstract: *Laboratory in vitro evolution (LIVE) might deliver DNA aptamers that bind proteins expressed on the surface of cells. In this work, we used cell engineering to place glypican 3 (GPC3), a possible marker for liver cancer theranostics, on the surface of a liver cell line. Libraries were then built from a six-letter genetic alphabet containing the standard nucleobases and two added nucleobases (2-amino-8H-imidazo[1,2-a]-[1,3,5]triazin-4-one and 6-amino-5-nitropyridin-2-one), Watson–Crick complements from an artificially expanded genetic information system (AEGIS). With counterselection against non-engineered cells, eight AEGIS-containing aptamers were recovered. Five bound selectively to GPC3-overexpressing cells. This selection–counterselection scheme had acceptable statistics, notwithstanding the possibility that cells engineered to overexpress GPC3 might also express different off-target proteins. This is the first example of such a combination.*

Researchers in biomolecular chemistry frequently need reagents that bind selectively to proteins, especially those on the surfaces of cells. Such reagents are very often antibodies. Aside from their biological complexity, antibody generation offers no easy way to generate a reagent that binds to the target in a cell-surface context. Therefore, alternative approaches have been suggested to replace antibodies as binding reagents. For example, libraries of DNA/RNA can be presented to a target protein displayed on cell surfaces. Library components that bind to the protein are recovered and PCR-amplified, perhaps with mutation, to give a daughter generation of "survivors" enriched in binding species. Repeating this cycle gives aptamers with improved binding in a process known as "in vitro selection",[1] "systematic evolution of ligands by exponential enrichment" (SELEX),[2] or, if mutation is involved, "laboratory in vitro evolution" (LIVE).[3]

However, aptamers have not replaced antibodies in cell biology, most likely owing to their poor affinities. These are often attributed to the limited repertoire of functional groups available on standard DNA/RNA, especially when compared to the repertoire available to proteins that contribute to binding.[3] For example, standard DNA/RNA has no positively charged groups (e.g., lysine and arginine), carboxylate groups (e.g., aspartate and glutamate), and a general-purpose acid–base catalyst (histidine) group, which are commonly found in the standard 20 amino acids.

Fortunately, synthetic biology has expanded the potential of LIVE by creating artificially expanded genetic information systems (AEGIS). AEGIS is a type of DNA/RNA with additional nucleotides and additional functionality. AEGIS rearranges hydrogen-bonding groups to allow DNA/RNA to contain as many as twelve independently replicable building blocks.[4] AEGIS supports six-letter LIVE (Figure 1A), where one of the added nucleotides (2-amino-8*H*-imidazo-[1,2-*a*][1,3,5]triazin-4-one, Z) has a nitro group, a universal binding unit that supports polyvalent interactions (compare nitrocellulose, for example). Previous studies suggest that this might increase the richness of an AEGIS library as a reservoir for binding aptamers, although direct comparisons are difficult to do.[3]

Herein, we asked whether AEGIS-LIVE might be expanded to target specific proteins in a cell-surface context. This would require that we a) engineer a recombinant cell so

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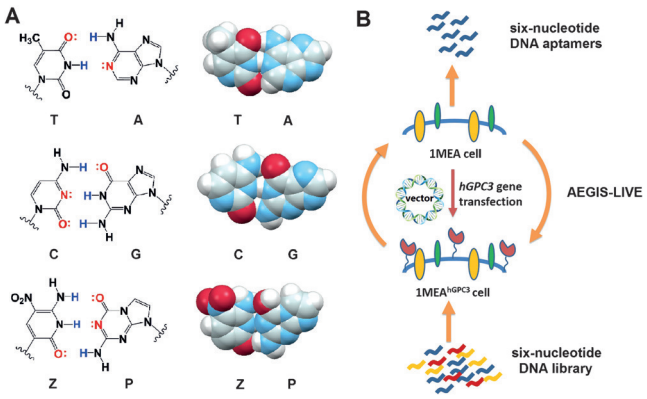


Figure 1. Chemical structures of the six nucleotides and AEGIS-LIVE. A) Molecular structures (left) and space-filling models (right) of C:G, T:A, and Z:P pairs showing their similarity (PDB ID: 4RHD). B) Engineering hGPC3-overexpressing cells and AEGIS-LIVE procedure.

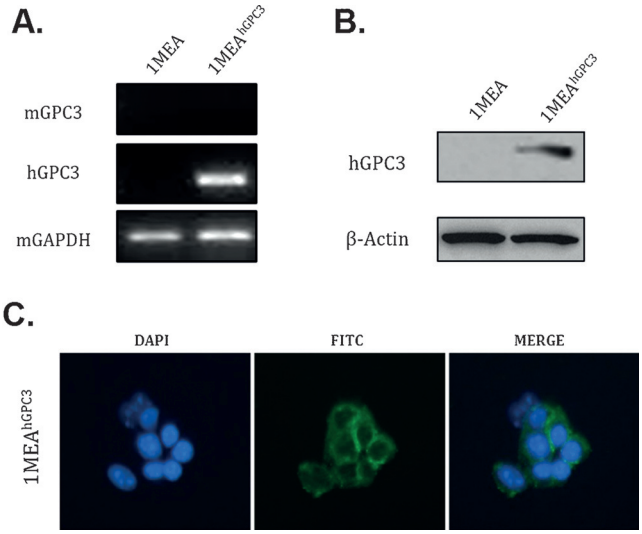


Figure 2. Confirmation of human GPC3 expression on the 1MEA cell surface in the engineered cells. A) RT-PCR assessing the hGPC3 gene transcription level after transfection. Mouse mGAPDH gene transcription was used as a positive control; mGPC3 was used as a negative control. B) Western blot showing the expression of hGPC3 after transfection. β-Actin was used as a loading control. C) ICC assays using the 1G12 antibody specific for GPC3, stained with a fluorescein-labeled secondary antibody. DAPI (blue) locates the nucleus, FITC (green) identifies antibody binding.

that it expresses the target protein abundantly on its surface, b) perform a cell-based LIVE using a six-letter GACTZP library (P = 6-amino-5-nitropyridin-2-one), and c) recover aptamers that bind to the cell for downstream analysis.

We have combined these three steps using glypican 3 (GPC3) as a protein target. GPC3 may be a specific biomarker for hepatocellular carcinoma (HCC).[5] Upregulated in most HCCs, it is absent from primary hepatocytes, cirrhotic cells, and benign lesions.[6] As liver cancer progresses, GPC3 may stimulate several oncogenic signaling pathways.[7] Monoclonal antibodies (mAbs) are known for GPC3, recognizing different epitopes on the core protein.[5a,c] Some are being examined as potential therapeutic agents through their binding to GPC3.[8]

These considerations suggest that AEGIS aptamers might also have clinical use, where intrinsic advantages of aptamers over antibodies might be exploited.[9] Aptamers (typically 10–100 nucleotides (nts)) are generally smaller than antibodies, easier to renature, cheaper to develop, cheaper to produce, and more stable to unfolding. Furthermore, facile reselection and combination afford DNA/RNA aptamers with increasing affinity, even in the nanomolar range, through SELEX/LIVE (Figure 1 B).

However, targeting cells that naturally express GPC3 would almost certainly generate aptamers that bind other surface proteins as well as those that bind GPC3. Whereas aptamers that bind off-target might be removed by a counterselection targeting the same type of natural cell lacking GPC3, it is hard to know where to find such a cell. Conversely, engineering cells to overexpress a target protein has a risk: While the engineering process can generally deliver a target protein to a surface, it might also alter other surface markers, with selection–counterselection strategies giving aptamers against other targets (Figure 1 B).

To explore the statistics of the cell-engineering strategy, we started with a murine cell line (BNL 1ME A. 7R.1; 1MEA) that does not express murine GPC3 (mGPC3; Figure 2), a cell useful for counterselection. We then transfected 1MEA with a plasmid carrying the human GPC3 gene (hGPC3) with a gene conferring neomycin resistance (NEO). Cells expressing hGPC3 ($1MEA^{hGPC3}$) were obtained by screening for resistance to the geneticin-selective antibiotic (G418 sulfate). hGPC3 expression was confirmed in mRNA (by RT-PCR) and protein (western blotting) levels. Immunocytochemistry (ICC) confirmed that hGPC3 is abundantly expressed on the surface of 1MEA engineered cells (Figure 2).

The GPC3-overexpressing $1MEA^{hGPC3}$ cells were then used as targets for AEGIS-LIVE, with 1MEA cells used for counterselection. A six-letter AEGIS-DNA library was synthesized from phosphoramidite mixtures (T/G/A/C/Z/P ≈ 1:1:1:1:2:2), with a 35 nt randomized region flanked by two 18 nt primer binding sites (see the Supporting Information, Table S1). The AEGIS-DNA library (20 nmol) was incubated with $1MEA^{hGPC3}$ cells (at 4 °C for 30 min). Unbound DNA oligonucleotides were then removed, bound ones were recovered, and a counterselection process (starting at round 5) was applied to the survivors. Following counterselection, survivors that bound GPC3-positive cells, but not 1MEA cells, were PCR-amplified[10a] (Table S2). Single-stranded AEGIS-DNA amplicons enriched in molecules having these dual binding features (Table S3) were recovered, and the selection cycle was repeated. Starting from round nine, two cycles of negative selection were conducted for each round of positive selection. Selection stringency was increased by using fewer cells and shorter incubation times. Enrichment of binding DNA oligonucleotides was monitored by flow cytometry with 5′-fluorescein tags (Figure S1).

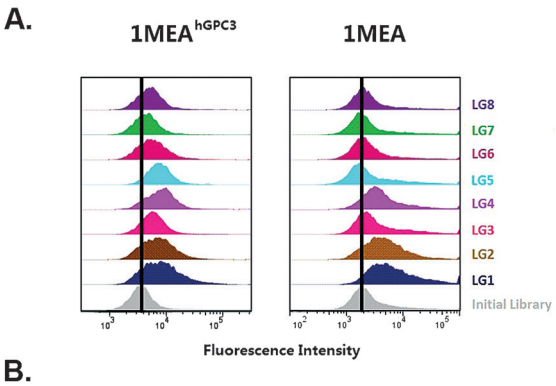
After eleven rounds, significant fluorescence was observed for GPC3-positive cells. In contrast, GPC3-negative cells showed little fluorescence. AEGIS-LIVE was stopped, and the survivor library from round 10 was PCR-amplified. The amplicons were then transliterated for six-letter AEGIS

deep sequencing [10] (Table S4). The eleven most abundant motifs contributed from 0.91 to 11.6% to the total survivor population (Table S5). These were resynthesized with 5′-biotin tags.

Binding of biotinylated aptamer candidates was compared for transfected 1MEA^{hGPC3} cells and untransfected 1MEA cells (30 min, 4 °C, 250 nM). A phycoerythrin (PE) streptavidin conjugate generated the observed fluorescence. The extent of cell labeling was determined by flow cytometry. Figure 3A shows the number of cells having the indicated level of labeling, the "effective number" of GPC3 sites per cell.

We then assessed the binding statistics, recognizing that the overexpression of GPC3 in the engineered cells might alter the level or kind of proteins displayed on their surface relative to the non-engineered cells. Interestingly, products **2**, **3**, **4**, **5**, **7**, **8**, **9**, and **11** (**LG1**–**LG8**, Figure 3B) all showed substantial binding to the engineered target cells. However, aptamers **LG1**, **LG2**, and **LG4** also showed binding to the untransfected "negative" cells. This thus provides a built-in metric for determining the success of the transfection–untransfection strategy. We hypothesized that **LG1**, **LG2**, and **LG4** bind to proteins other than GPC3 that were presented by the engineered cells, perhaps in amounts different from the surface of non-engineered cells, which might be a consequence of the engineering.

We then tested the ability of GPC3 cell-specific aptamers to bind other cells, including the hGPC3-positive human liver cancer cell lines HepG2, Huh7, and Hep3B, the mGPC3-positive liver cancer line Hepa1-6, the hGPC3-negative human liver cancer lines LH86 and SK-Hep1, and the hGPC3-negative non-cancerous human liver cell line Hu1545 (Figure S2). Aptamers **LG5**, **LG6**, and **LG7** showed substantial binding to all four GPC3-positive cells. Aptamers **LG3** and **LG8** showed binding to HepG2, Hep3B, and Hepa1-6 cells. However, **LG3** and **LG8** were not observed to significantly bind HuH7 cells (Table 1). None of the five aptamers bound to GPC3-negative SK-Hep1, LH86, and Hu1545 cells.



B.

Name	Sequence	Percentage
LG1	~PGGTGGGCGGAGGTCTZGCTACAPGPTTTGGPGGC~	11.37%
LG2	~PGCCCGGGPTAPPGTGPTGGGTGTTCGCTATCCAG~	7.98%
LG3	~GGTAACTAGTAGTTGACCCTGPAGTGZTGTPTCTG~	6.01%
LG4	~GGCGGGGTZGPGTAAGGGGTCTAAGGCATTGGGTC~	4.48%
LG5	~GGAGGAAGTGGTCCTTGCTTTGCZTCGTATCTGGG~	2.57%
LG6	~GGTZGATTATTPGGTTCAATAACACPTCCTGGTGG~	1.96%
LG7	~PGCACAGTGTGZZCCATAGGTTGTAATGACPTZTG~	1.04%
LG8	~GGCAGCZCCTGPAGTPGAGTGTPATGGCTTATTCG~	0.91%

Figure 3. Names, sequences, pool percentages, and specificities of selected aptamers. A) Binding of DNA aptamer candidates. Left: Binding to positive 1MEA^{hGPC3} cells in the selection. Right: Binding to negative (original) 1MEA cells. The gray distributions were generated with the initial DNA library. B) Sequences (randomized region) and their percentage contribution to the pool of surviving binders (AEGIS Z and P are shown in red and blue, respectively).

Table 1: Binding profiles of selected aptamers on seven cell lines.[a]

	hGPC3-positive cells				hGPC3-negative cells		
	HepG2	Hep3B	HuH7	Hepa1-6	SK-Hep1	LH86	Hu1545
LG3	+	+	−	+	−	−	−
LG5	++	++	+	+	−	−	−
LG6	+	+	+	++	−	−	−
LG7	++	+	+	+	−	−	−
LG8	+	+	−	+	−	−	−

[a] The threshold based on the fluorescence intensity of PE in the flow-cytometric analysis was chosen so that 95% of the cells incubated with the PE-labeled unselected DNA library would have lower fluorescence intensity than the threshold. When the PE-labeled aptamers were allowed to interact with the cells, the percentage of cells with fluorescence above the set threshold was used to evaluate the binding capacity of the aptamer to the cells. 0–10%: −; 11–30%: +; 31–50%: ++.

Next, the apparent dissociation constants (K_d) for HepG2 cells were estimated for individual AEGIS aptamers using different concentrations (0.1–1000 nM) of biotinylated aptamers, with fluorescence coming from PE conjugated with streptavidin. The best aptamer, **LG5**, had a K_d value of 6 nM. The other K_d values were in the range of 60–500 nM (Figure S3). To show that the AEGIS nucleotides were essential for binding in **LG5**, their analogues of replacing Z with A, T, G, C were tested, and none of them showed any binding (Figure S4), demonstrating the significant role of Z in aptamer **LG5**.

We then asked whether these aptamers would compete with an anti-hGPC3 antibody generated against hGPC3. Here, high concentrations (2.5 μM) of the aptamer were incubated with HepG2 cells followed by incubation with the antibody (250 nM). The aptamers were not displaced by the antibodies (Figure S5). This is perhaps not surprising as the details of aptamer selection and antibody selection are sufficiently different that one might not expect the two processes to find the same epitopes.

To obtain further evidence that the selected aptamers bind to surface GPC3 (Figure 4A), the hGPC3-positive HepG2 cells were lysed, biotinylated aptamers were added in parallel with a biotinylated random DNA sequence (RS), and the biotinylated aptamers (RS)/lysate complexes were recovered on streptavidin-modified magnetic beads. Recovered protein was analyzed by SDS-PAGE and western blotting with anti-hGPC3 mAb. Figure 4B shows that **LG5**, **LG6**, and **LG7** captured hGPC3 from HepG2 cell lysate. Some nonspecific binding was also seen between RS and hGPC3. **LG3** and **LG8** showed no selective binding, which is possibly due to their higher dissociation constants (Figure S3).

AEGIS-LIVE is in its infancy to create ligands for proteins "on demand". Much work remains to be done to

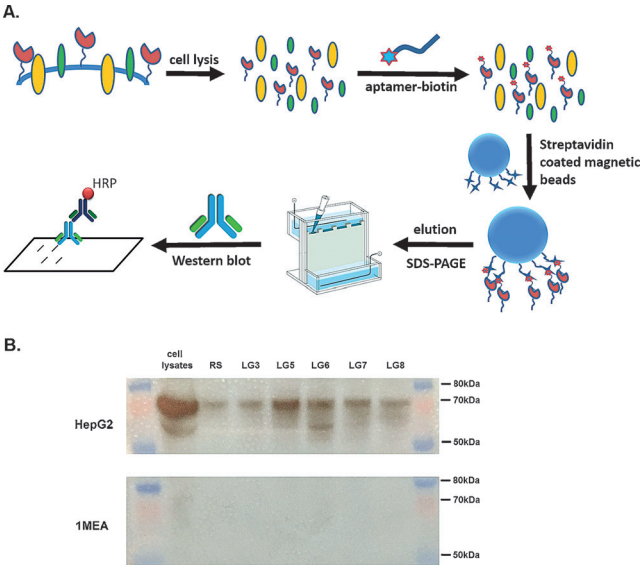


Figure 4. Target confirmation of selected aptamers. A) Procedure to identify the target of aptamer candidates. B) Western blot assay of biotin–aptamer–target complexes captured by streptavidin-modified magnetic beads using anti-hGPC3 primary antibodies.

show how generally the added information density and added functionality of AEGIS libraries can improve their richness as reservoirs compared to standard libraries. Adding functionality alone appears to have worked for expanding SELEX as done by SomaLogic, where all four standard nucleotides carry functionality to give SomaMers.[11] Click-SELEX is also an elegant way to add functionality.[12] These methods do not, of course, have additional information density. However, work by Hirao and co-workers with extra nucleotides does expand the alphabet, and also appears to improve aptamer performance.[13]

From a technical perspective, we believe this to be the most successful AEGIS-LIVE system reported to date. Given the novelty of the AEGIS nucleotides and that polymerases have a propensity to reject unnatural nucleotides, it remains a challenge to retain them through many PCR cycles. Here, however, retention was the highest yet seen, perhaps because of improved AEGIS PCR or perhaps because of the higher amounts of AEGIS nucleotides in the original library. Furthermore, these results confirm that aptamers containing Z and P can have excellent affinities in the nanomolar range, the range of the very best GACT aptamers.

In summary, a series of six-nucleotide aptamers have been found to target cells engineered to overexpress a specific cell-surface protein, with the non-engineered cell serving in a counterselection. These aptamers could be used to distinguish cells that display that protein from those that do not, and might thus become useful for hGPC3 detection and therapy.

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