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GRANT NUMBER DAMD17-97-1-7310

TITLE: Breast Tumor Detection Utilizing Novel in situ Amplication Systems

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (7 Jul 97 - 6 Jul 98)

4. TITLE AND SUBTITLE

Breast Tumor Detection Utilizing Novel in situ Amplication Systems

5. FUNDING NUMBERS

DAMD17-97-1-7310

6. AUTHOR(S)

Cassandra L. Smith, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Boston University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The long term goal is to construct *in situ,* at the site of a tumor. The aggregate will be formed by the sequential administration of small molecules, then a radioactive label will also be delivered by a small molecule. Thus, the tumor to normal tissue radioactivity ratios should be exceptionally high for both diagnostic and therapeutic purposes. The amplification system will consist of two or more classes of multivalent molecules, A, B, C that cannot self assemble but can associate very specifically with each other. This work will take advantage of the known high specificities of single-stranded DNA to bind its complement to form double-stranded structures, of streptavidin to bind biotin, and of pairs of leucine zipppers to dimerize. This project will focus on optimizing the method in model systems and on tumor cells in culture.

14. SUBJECT TERMS
Breast Cancer
Tumor detection and tumor cell aggregation signal amplification.

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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8/1/97

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Introduction

In recent years considerable interest has been generated in the use of anti-sense oligonucleotides and single-stranded DNA for *in vivo* therapeutic applications (1-3). Exciting examples include selective inhibition of viral RNA replication and gene expression (4,5). The application of oligonucleotides into clinical medicine was encouraged by the recent study of Kulka *et al.*(6) which reported successful inhibition of viral gene expression *in vivo* by anti-sense DNA. Additional *in vivo* applications have included oligonucleotide-antibody conjugates for tumor diagnosis and therapy (7).

Others have investigated in animal models the pharmacokinetics, biodistribution and stability of of oligonucleotides ranging in size from 12 bases (8) to as large as 38 bases (9), and labeled with radioisotopes, such as ^{125}I (7,10,11) ^{32}P (9,12), ^{3}H (8) ^{35}S (12-14) and ^{111}In (15,16). In these and other *in vivo* applications the nucleotide backbone was modified to stabilize the molecule against nuclease digestion, typically with methylphosphonates (17,18,19,20) or phosphorothioates (21-24). However, terminal modification alone was shown to provide sufficient stability *in vivo* (9,13,20,25).

Amplification techniques for nucleic acids, are under active investigation for *in vitro* applications. These include PCR (26) the "christmas tree" approach (27), and branching nucleic acid dendrimers (28-34). However, using nucleic acids for amplification *in vivo* has not previously been considered.

The overall goal of this project is to form at the site of a tumor a large aggregate. The aggregate is formed *de novo* from a set of small molecules with do not self aggregate but can associate with each other. The formation of the aggregate means that the original tumor target signal is amplifed. Then, the aggregate at the site of the tumor is used as the target of a small molecule carrying a low molecular weight label such as an imageable radionuclide. This proposal describes the assembly *in situ* of an aggregate possessing what could be an almost limitless amount of radioactivity while at the same time the method seeks to minimize radioactivity in normal tissue. Thus, the tumor to normal tissue radioactivity ratios should be exceptionally high for both diagnostic and therapeutic applications.

This approach is based upon several well characterize high affinity systems (double-stranded DNA, streptavidin-biotin, and leucine zipper dimerization) that are being combined in a unique fashion. Nucleic acids and their derivatives were chosen because (1) the affinity and specificity between two complementary DNA sequences in natural settings is quite efficient and tight, (2) short DNA molecules should be non-toxic and (3) DNA molecules can be radiolabeled relatively easily via a number of strategies. A streptavidin-biotin system was chosen because of (1) the high stability of this protein *in vitro* and *in vivo* and its low immunogenicity, (2) the existence, and ongoing construction, of a variety of potentially useful recombinant streptavidins (3) the ease of biotinylation of a wide variety of molecules. The dimerizing leucine zipper recombinant contructs was choosen because of (1) the specificity and stability of the dimer and the fact that the zipper can be formed with a short oligopeptide. The three novel amplification strategies to be tested here are (a) a protein-nucleic acid aggregate consisting of pairs of streptavidin jonjugated with complementary single-stranded DNAs (PNAs), (b) a pure nucleic acid aggregate consisting of sets of branches double-stranded nucleic acids with single stranded ends aare are complementary to the ends of other member of the set and (c) a pure protein aggregate consisting of pairs of stretavidin fused to the ends of leucine sippers. One zipper is fused to the

N-terminus of streptavidin and the other is fused to the C-terminus. It is also anticipated, that the nucleic acid amplification schemes described herein will have applications which extend far beyond the intent of this proposal.

This project will test and optimize novel amplification schemes *in vitro* (in solution, in serum and in breast cancer tumor cell cultures). If time permits the optimized schemes will also be tested, *in vivo*, in a mouse model (using an immobilized artificial target and/or a breast tumor xenograft).

Technical Objectives: This research will use two primary target systems: (a) artificial tumor mimics in solution (and in serum and tissue culture medium) and (b) immobilized breast cancer tumor cells in culture. If time permits , test systems in mice using (if necessary, tumor mimics an artificially immobilized target) and a breast tumor xenograph will be tested. The targets will be tested with three types of amplification systems: (1) a protein-nucleic acid complex, (2) a pure nucleic acid complex and (3) a pure protein amplifcation system (described above).

Body

Materials: (A) Nucleic Acids: Oligonucleotides (modified with biotin or flourescent labels) were purchased from Operon Technologies (Alameda, CA). The initial experiments will use DNAs but the ultimate system will incorporate PNAs, nucleic acids with polypeptide backbones (35). PNAs bind complementary single-stranded DNAs with an affinity that is greater than that seen with double-stranded DNAs. PNAs are resistant to degradation by nucleases, do not appear to be bound by serum proteins which bind DNA, and appear to have better biodistributions than equivalent oligonucleotides. Thus, it is our intent to use these nucleic acids analogs *in vivo*. Although these molecules are available commercially (PerSeptive Biosystems, Boston) these molecules will not be used initially to set up and test the aggregation system because DNA oligomers are considerable cheaper (hence, allowing the exploration of the size and sequence dependence of the aggregates) and are easily manipulatable with a large number of enzymes. These experiments will also explore well-characterized dedrimeric (36) systems available commercial (Polyprobe, Philadelphia) or described by others. The nucleic acids will flourescent labeled for direct imaging of aggregates with a flourescent digital microscope or with ^{32}P (an easy to work with label) using conventional methods. Eventually, the in *vitro* and *in vivo* test system will use the imageable radionuclide, ^{99m}Tc, introduced covalent linkage of the nucleic acids with chelators (37).

(B) Streptavidin: Streptavidin is a 60 kDa tetrameric protein which binds four biotins (or biotinylated macromolelcules like proteins or nucleic acids) with a K_d of 10^{-15} M. This means that binding occurs spontaneously and irreversibly. Flourescently labeled biotin, nucleic acids or streptavidin are used to assess aggregation.

Methods: A general approach to *in vivo* targeting is illustrated in Figure 1 for the protein-nucleic acid construct. The first step involves the administration of a breast tumor-specific molecule tagged with a link a linker to the aggregation system. This could be an antibody directly conjugated directly with a single-stranded oligonucleotide using methods we have developed or with biotin. This is followed by the administration of a streptavidin conjugate containing four identical biotinylated single-stranded DNA molecules, each 20 to 24 bases in length.

Alternatively, this construct could preseve one of the four biotin binding sites for binding to a biotinylated antibody. Next a second streptavidin complex is added. This streptavidin contains four single-stranded DNA molecules complementary to the single strands of the first complex. Thereafter, alternating injections of complementary oligonucleotides should produce a large protein-DNA aggregate at the site of the tumor. Finally, the radiolabel will be injectied in the form of a radiolabeled single-stranded DNA, complementary to most recently injected oligonucleotide.

Potentially each cycle of addition of complementary DNA strands, for tetra-valent reagents, will result in up to a three-fold increase in the number of strands added to the aggregate. This amplification is potentially greater than that of PCR which makes it an attractive candidate for *in vitro* applications as well. Using these methods it should be possible to construct an aggregate at each antigenic site, which contains an exponential increase in the amount of radiolabel achievable by direct labeling techniques. Moreover, the radioactivity should be concentrated at the tumor due to the quick clearance of the low molecular weight radiolabeled strands from circulation and normal tissues.

b. establish breast tumor cell lines
c. purify needed reagents (streptavidin, nucleic acids, dendrimers etc.)
d. initialize experiments on tumor cell mimic

Results: A very simple system was set up to study layer formation. In this system the build up of the aggregation was followed on a flat surfaces. This model system allows the formation of an aggregate in two dimensions. In this case floresecently labeled Streptavidin was used to follow the formation of the aggregation. One might envision surch a surface as the outside layer of a tumor. The results of such an experiment are shown in Figure 2. The data shows that the use of a 50-mer linker easily allowed four layers to be built up. At this time it is not clear why the aggregation size could not be extended beyond four layers but there are many variable to be tested. For instance, it may be that the geometry of the biotinylated DNA strands emerging from the biotin binding site of Streptavidin sterically hinder the construction of more layers.

Another simple system that formed a three dimensional structure was also studied. This system used streptavidin coated beads. The beads were divided into two batches and complementary DNAs (about ~1000 base pairs in length) were bound to the separate batches. No aggregation was detected when the two batches of beads with complementary DNAs were mixed together. However, when the complementary DNAs were first hybridized to themselves and then mixed with beads large aggregations formed (Figure 3). On average, the aggregations were 4 beads in diameter. This was consistant with the results obtained with the two dimensional structures. It is likely that secondary structure of the ~1000 base single strands interfered with their hybridization.

There are an infinite combination of DNA sequences and length that could be tried to form aggregates. Our focus will first be on defining minimal length of DNA that can be used and will likely used the prehybridized bis-biotinylated double stranded DNA to test different length of DNA sequence. This focus is because we would like to work with the smallest length single stranded DNAs that we can to avoid secondary structure.

Experiments have begun on established breast tumor cell lines. The first question that is being asked is what happens to DNAs on the surface of tumor cells. In our initial model system, breast

tumor cell line MC-7 is being biotinylated and streptavidin will be added to surface to serve as a target for DNA.

Conclusions: Two simple model systems have been implemented to study aggregate formation. Both system form aggregates using a streptavidin-biotinlyated DNA complexes. One sytem forms a two-dimensional aggregate and the second system forms a three-dimensional aggregate. Aggregate formation has been demonstrated with both system. However, the aggregation limit appears to be about four layers. This may be due to the impurities of the reagents or steric limitation imposed by the geometry of the biotin binding sites of Streptavidin.

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Figure 1. Schematic of aggregates formed using Streptavidin-biotinylated DNA linkers (see text for details).

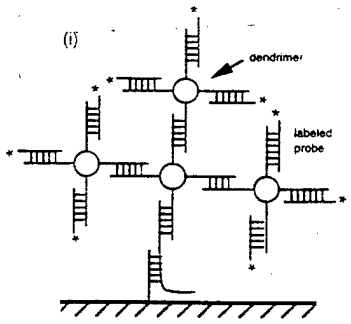

Figure 2. Formation of Streptavidin-Biotinylated DNA Aggregates on Silicon Surfaces (see text for details)

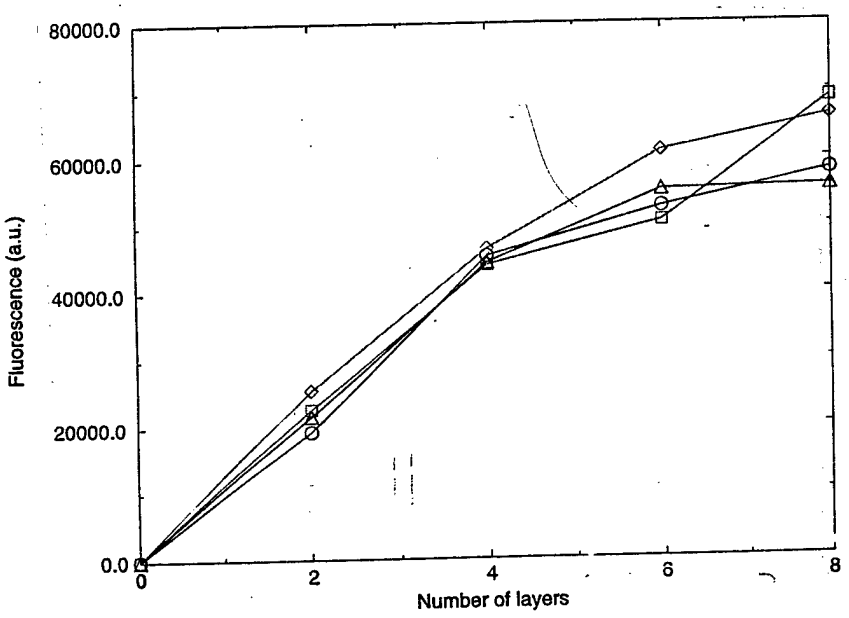

Figure 3. Formation of Streptavidin Coated Microbeads and Biotinylated DNA Aggregates in Solution. (A) Aggregation of streptavidin coated microbeads through a bis-biotinlylated double stranded DNA. (B). Beads with non-complementary DNAs do not agggate.