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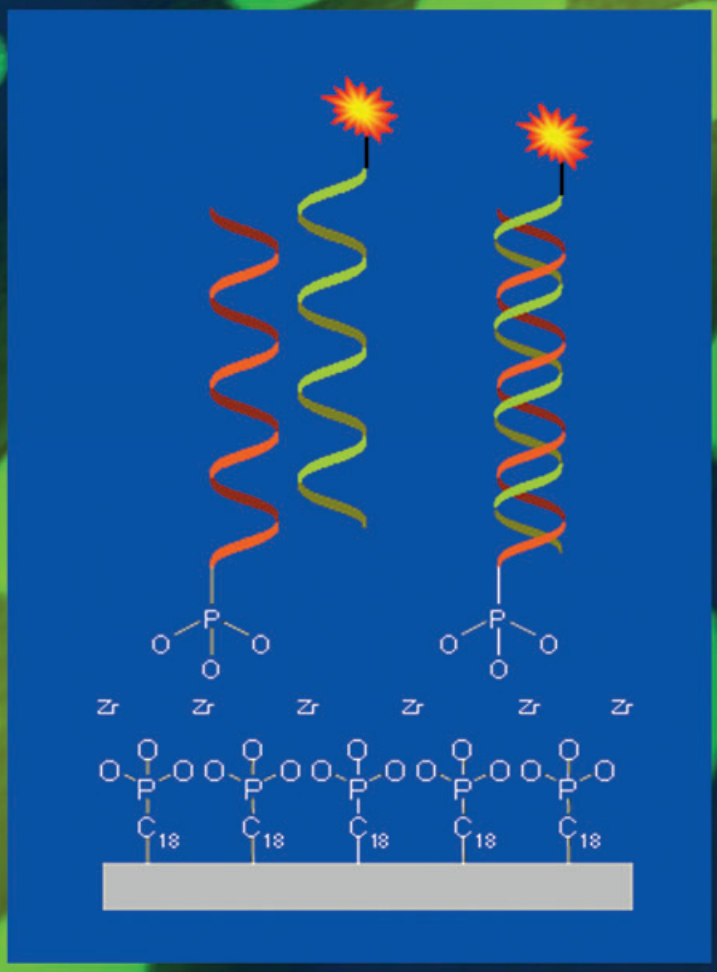

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Metal Phosphonates Applied to Biotechnologies: A Novel Approach to Oligonucleotide Microarrays

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Abstract: A new process for preparing oligonucleotide arrays is described that uses surface grafting chemistry which is fundamentally different from the electrostatic adsorption and organic covalent binding methods normally employed. Solid supports are modified with a mixed organic/inorganic zirconium phosphonate monolayer film providing a stable, well-defined interface. Oligonucleotide probes terminated with phosphate are spotted directly on to the zirconated surface forming a covalent linkage. Specific binding of terminal phosphate groups with minimal binding of the internal phosphate diesters has been demonstrated. The mixed organic/inorganic thin films have also been extended for use arraying DNA duplex probes, and therefore represent a viable general approach to DNA-based bioarrays. Ideas for interfacing mixed organic/inorganic interfaces to other bioapplications are also discussed.

Keywords: DNA · microarrays · monolayers · oligonucleotides · phosphonates

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Introduction

DNA arrays have emerged as a convenient and powerful tool for highly parallel, high-throughput experimentation in molecular biological research.[1–6] Typical arrays (Figure 1)

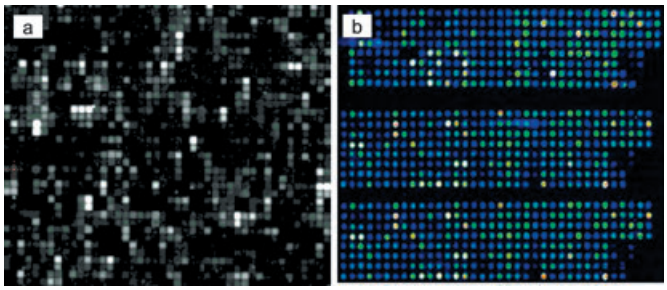

Figure 1. Examples of a) in situ (Affymetrix) and b) spotted arrays. In a) features are approximately 25 microns, while in b) the spots are 150 microns in diameter. (Image a was provided by the joint Shands Cancer Center/Interdisciplinary Center for Biotechnology Research at the University of Florida.)

consist of either single-stranded or double-stranded products of different sequences, called probes, that are bound to a surface and are available for subsequent complexation by targets, leading to a signal that is detected by an imaging technology, most often fluorescence. Applications of arrays that take advantage of interactions between complementary single-stranded DNA (ss-DNA) include rapid and accurate gene mapping, DNA sequencing, mRNA expression analysis, and diagnosis of genetic diseases. DNA arrays can also be used to investigate DNA-protein interactions leading to analysis of transcriptional control, identification of individual contacts between nucleotides and amino acids, detection of differences in binding specificity among proteins in the same family, and determination of DNA-binding specificity for uncharacterized proteins. Biochip technology is having a major impact in biological research and drug discovery programs by simplifying the detection of biological residues

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with the advantage of using very small amounts of biological probes (from nanomoles to picomoles), along with the capability of analyzing thousands of analytes in parallel.

Two general processes have established themselves for producing DNA arrays.[4,6–16] In situ synthesis[9–13] can lead to oligonucleotide arrays with extremely high complexity, containing hundreds of thousands of different probes on a single array.[14–16] An example (Figure 1a) is the Affymetrix method of microarray production, which involves base-by-base synthesis using photoremovable protecting groups.[17] While very successful, these arrays are not suited for all applications. Expense and availability limit their accessibility, and there are also some practical limits to the length of the oligonucleotide probes, because the synthetic cycle yields, while good, are less than 100%. The other popular approach to microarrays involves "spotting" techniques that use automated robots to array oligonucleotides previously synthesized by chemical or enzymatic methods.[6,18–29] Spotting methods are limited to lower array complexity, 10000 or fewer spots per array (Figure 1b), but permit more flexibility in choice of probes. For example, probes can range from short oligomers to long pieces of DNA obtained from clone libraries or by polymerase chain reaction (PCR).[4]

Glass substrates are preferred for arrays, offering advantages over other options.[30] Glass is flat and nonporous, so the hybridization volume can be kept to a minimum, and it is durable under the temperatures and chemical conditions normally employed. Furthermore, the low fluorescence of glass does not significantly contribute to background noise when fluorescence is used for detection. However, oligonucleotides bind poorly to glass, so some surface derivatization is required. The simplest binding mechanism is electrostatic adsorption of probes onto a charged priming layer, such as a polylysine film or an aminosilane layer (Figure 2).[18,19] The

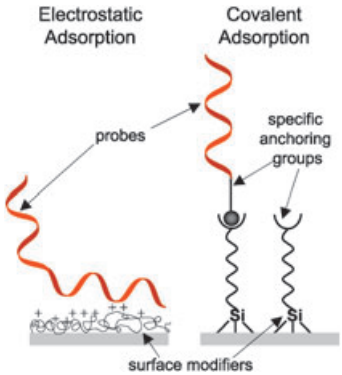

Figure 2. Scheme illustrating electrostatic and covalent immobilization of oligonucleotide probes.

potential drawback is that electrostatic binding encourages the probes to lay flat on the surface, reducing hybridization efficiency. An alternative is to form a surface bound monolayer of functional groups that are available to react only with a specific group on the probe terminus, resulting in covalent linkage (Figure 2). Organosilane-coating protocols are commonly used to fix the active groups to the glass surface. Some combinations of surface/olignucleotide function that have been demonstrated include thiol/acrylamide,[20] activated carboxylic acid/amine,[21,22] amine/aldehyde,[23–25] epoxide/amine,[26] aldehyde/oxyamine,[31] and biotin/streptavidin.[27–29]

The purpose of this Concept article is to highlight a fundamentally different route for covalently attaching DNA probes to surfaces for array applications. The new approach uses a mixed organic/inorganic monolayer to derivatize the glass and generate a reactive surface (Figure 3). Probe at-

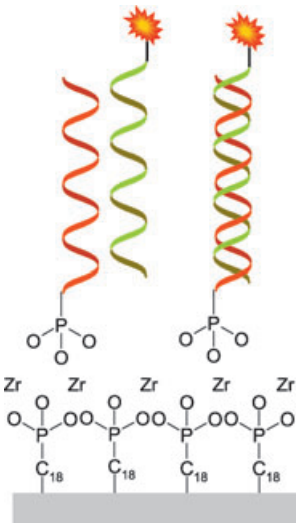

Figure 3. Organic/inorganic surfaces for DNA arrays. Phosphate terminated probes attach specifically via coordinate covalent bonding (bonds are left out of the figure for clarity) to a zirconated phosphonic acid modified surface.

tachment is then through a highly specific coordinate covalent linkage between a terminal phosphate group on the probe molecules and the inorganic ions on the glass surface. An advantage over other methods currently in use is that phosphate is a naturally occurring function that does not alter the intrinsic nature of the probe, and it can be introduced chemically or with enzymatic routes, offering the possibility of using PCR products as starting materials. Furthermore, the DNA grafting process is simple, performed in a single step instead of multiple chemical coupling reactions.

Oligonucleotides Binding on Inorganic Surfaces

DNA interactions with inorganic surfaces have previously been exploited. In the 1980s, information about conformational details and helical periodicity was obtained by adsorbing the DNA onto an inorganic surface before enzymatic or chemical digestion.[32,33] The adsorbed DNA helix is thus hindered in a uniform way along its length and only those

bonds of the DNA backbone most exposed to solution are fragmented. The resulting modulated cutting pattern is directly translated into the periodicity of helical twists. In these[32,33] and other[34] examples of DNA binding to inorganic surfaces, it is the polymer backbone that interacts with the metal centers, either electrostatically or through binding of the phosphate diester groups.

For DNA array applications, specific attachment of the probe molecule at one of its ends is preferred in order to keep the backbone free to maximize the chance of interacting with a target, but there are few examples of specific attachment of a DNA terminus to an inorganic surface. The principal exception is binding oligonucleotides to metallic gold with the same sulfur–gold linkages used to prepare self-assembled monolayers (SAMs).[35–38] Surface plasmon resonance (SPR) is currently the main application of gold-immobilized DNA.[38,39] SPR can be used to monitor mass changes associated with the probe immobilization event itself, or as a very sensitive detector of complexation. Gold surfaces can be used for DNA or protein array applications by using powerful SPR imaging methods,[35,40] although this promising method is not yet widely available.

Metal Phosphonate Modified Slides

For a modified surface to be useful as a substrate for DNA arrays, it should terminate in a group that is compatible with an easily prepared end group on the probe molecules, involve chemistry that is easily transferable to glass, and offer low background noise during detection. An inorganic system that we have found to meet these requirements is a series of thin films of metal organophosphonates and organophosphates.[41–47] In the solid-state, many examples form layered structures with organic sheets separating continuous inorganic networks of metal ions coordinated by the phosphonate or phosphate oxygen atoms.[48] An example is the layered $Zr(O_3PC_6H_5)_2$ shown in Figure 4.[49] In 1988, Mallouk and co-workers[50] demonstrated that the same metal phosphonate interactions that hold the layered solids together could be used to prepare multilayered thin films of α,ω-bisphosphonates by sequentially adsorbing the metal ions, usually Zr^{4+}, and the bisphosphonic acid onto a flat surface. This chemistry has been used to prepare organized organic films for topics that include nonlinear optics, electron transfer, and electrochemical sensing.[34,51–54]

While multilayered structures can be achieved by cycling the alternate layer process, an active surface is generated by binding Zr^{4+} ions to a phosphate or phosphonate monolayer, as indicated in the scheme in Figure 5. This surface can subsequently be used to adsorb another layer of an organophosphonate or phosphate that deposits as a type of self-assembled monolayer.[41] Our groups have used this chemistry to form monolayer films of organophosphonates with organic groups ranging from simple alkanes[41] to azobenzene,[45] tetrathiafulvalene,[55] phenoxy and biphenoxy groups,[43] and porphyrins.[46]

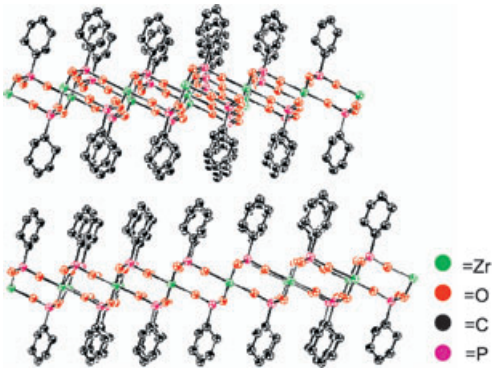

Figure 4. Cross section of the layered structure of $Zr(O_3PC_6H_5)_2$. The same Zr^{4+}/RPO_3^{2-} bonding present in the solid-state structures contributes to the stability of the monolayer films. The figure was drawn from crystallographic data from reference [49].

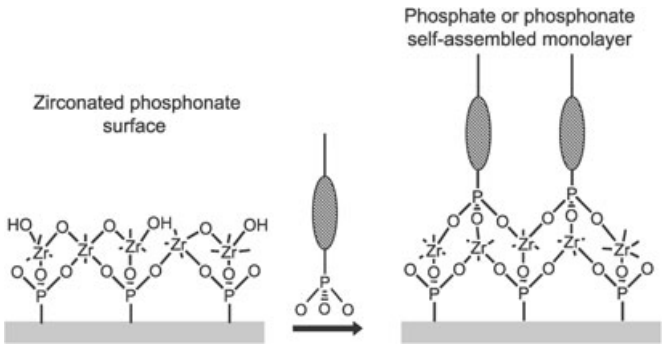

Figure 5. Adsorption onto a zirconated phosphonic acid surface to form a self-assembled monolayer of a functionalized organophosphonate. Under aqueous conditions, the coordination sphere of the Zr^{4+} ions is initially completed with oxide and hydroxide ions (left), so the surface is charge neutral, minimizing nonspecific electrostatic adsorption. Terminal phosphonate or phosphate groups are basic enough to compete for the zirconium ions to complete the zirconium phosphonate (or phosphate) bilayer (right).

The zirconium phosphonate-modified surfaces can be prepared in different ways, but often involve binding of Zr^{4+} ions to phosphorylated groups deposited onto silica[50,51,54,56–61] or gold (Figure 6).[52,54,56,57,62] Our experience is that exceptionally smooth and uniform films can be gener-

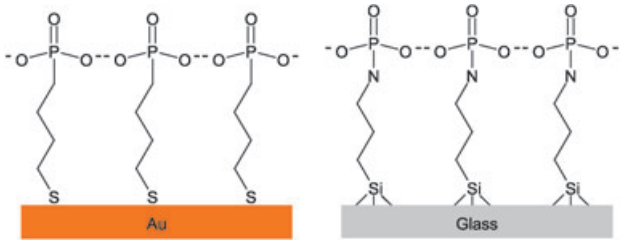

Figure 6. Examples of covalently adsorbed phosphonate films.

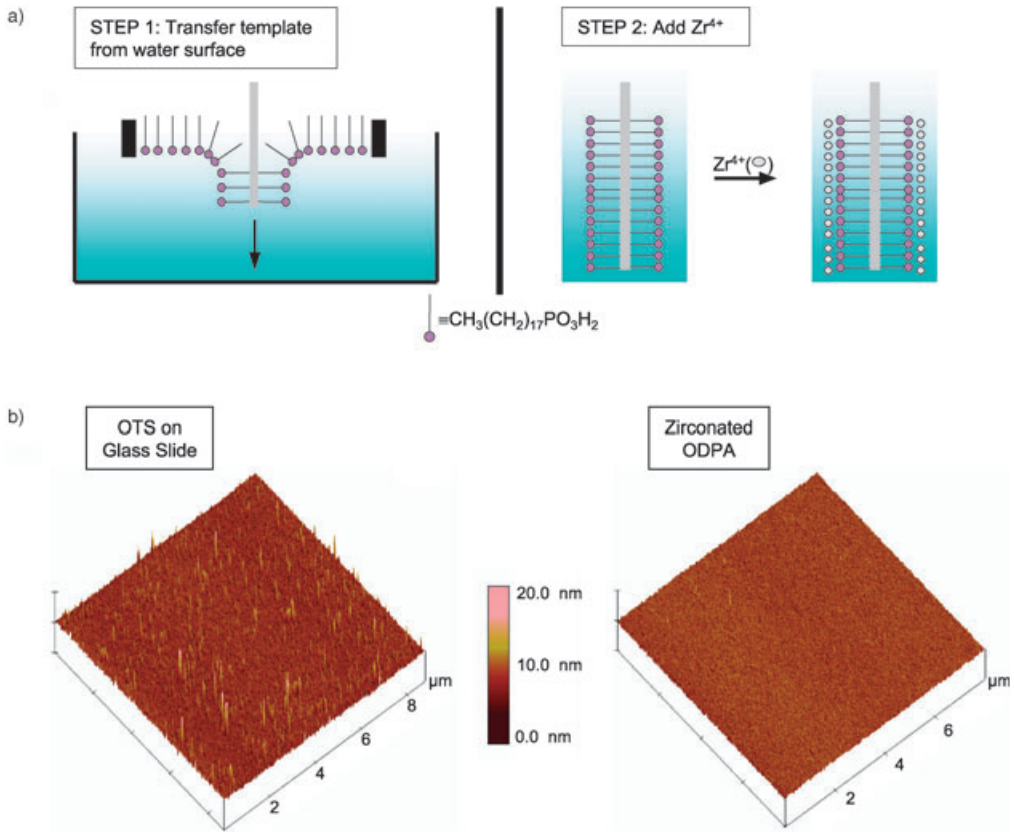

Figure 7. Langmuir–Blodgett route to zirconated phosphonate modified slides. a) In step 1, a monolayer of octadecylphosphonic acid (ODPA) is deposited onto a hydrophobic slide (here, made hydrophobic with octadecyltrichlorosilane, OTS). In step 2, the slide is then exposed to a solution of $Zr^{4+}_{(aq)}$. The slide can then be rinsed and used immediately or stored in water for months before use. b) The zirconated ODPA slides are very smooth (right), compared here to the OTS covered glass before deposition (left).

ated on hydrophobic supports by using Langmuir–Blodgett (LB) methods.[41–46] The LB process begins with an octadecylphosphonic acid (ODPA) Langmuir monolayer that is deposited onto the hydrophobic solid support in such a way that the hydrophilic acid group (PO_3H_2) is directed away from the support (Figure 7).[41,42] The substrate is then removed from the LB trough and exposed to a solution of Zr^{4+} ions that bind to give a monolayer of the zirconated octadecylphosphonic acid (ODPA-Zr). In solid-state zirconium phosphonates, each Zr^{4+} ion is coordinated by oxygen atoms from different molecules, thus linking them together. The same situation arises in the zirconated LB films. The strongly binding zirconium ions cross-link the original monolayer, providing a well-defined interface of zirconium phosphonate sites[41,42] that sticks strongly to the surface, because it is no longer a traditional LB film of individual molecules physisorbed to the surface but rather a network or monolayer tape in which adhesion comes from the sum of all molecules in a cross-linked array. The zirconium phosphonate films are not soluble in organic solvents, and dissolve in water only below pH 1. Our experience is that glass slides coated with the ODPA-Zr monolayers can be stored in water for months and retain activity with no evidence of desorption.

Metal Phosphonate DNA Arrays

The key to binding DNA to the zirconated surface is to terminate the probe strands with phosphate groups, which is routinely achieved using enzymatic (T4 polynucleotide kinase) or chemical (phosphoramidite chemistry) routes. For this to work, the free phosphate group introduced on the 5′ end of oligonucleotides needs to dominate over the backbone phosphate diester groups for binding to the zirconium ions on the surface. A hint that this will be the case comes from previous studies of native double-stranded DNA (ds-DNA) samples adsorbed to aluminum alkanebisphosphonate thin films. Mallouk, Bard, and co-workers[34] used electrogenerated chemiluminescence to detect binding of calf thymus ds-DNA at electrodes coated by the multilayer metal bisphosphonate sequential deposition process, described above. The authors observed significant DNA adsorption when Al^{3+} ions were used, but a much smaller extent of immobilization when films were prepared with Zr^{4+} or La^{3+}. Presumably, the native ds-DNA adsorbs through the phosphate diester groups of the backbone, and these observations suggest that this interaction is much weaker with zirconium ions. In contrast, we and others have previously observed that molecules having terminal phos-

phonate or phosphate groups bind very strongly to zirconated monolayer films.

Direct evidence for specific binding of the probe terminus to the surface comes from competition studies between a probe containing a 5′-phosphate group and a probe of the same sequence without it.[47] Table 1 shows the results of this

Table 1. Fluorescence intensity at 5′-phosphorylated and nonphosphorylated probes after hybridization with the corresponding Cy-3 labeled complement.

Concentration of spotted oligo [μM]	O33(X)[a]	5′H_2O_3PO-O33(X)
50	4300	27000
20	3900	26000
5	2200	15000

[a] O33(X) corresponds to a 33 mer oligonucleotide.

study for which there was 6–7-fold more binding of fluorescently labeled target molecules at the phosphate-terminated probes, indicating that these probes bind more strongly to the zirconated surface. Furthermore, they are not removed by the hybridization and rinsing procedures.

This trend is significantly enhanced if a passivation step is added to inactivate unspotted regions of the array. While nonspecific binding of the unmodified probes is small, it is still measurable. The same interactions with the surface will be present with fluorescently labeled targets, leading to significant background. This background can be reduced by treating the slides with BSA (bovine serum albumin) after spotting. The BSA passivates unspotted regions. However, we have also observed that the BSA treatment displaces probes that are physisorbed, while leaving behind the covalently attached probes. Therefore, when the BSA treatment is included during the comparison of target binding at modified versus unmodified probes, the intensity ratio approaches 1000 (Figure 8).

The strong binding of the terminal phosphate groups and the weak interactions of the polymer backbone with the zirconated surface can be understood by realizing that under aqueous conditions the zirconium layer is terminated in oxide and hydroxide ions (Figure 5). In the pH range used for most bioarray applications, this surface will be charge neutral, so electrostatic adsorption of the charged oligonucleotide will be minimal. This situation is in marked contrast to the amine-terminated slides commonly in use, which are protonated and charged under these conditions, leading to electrostatic adsorption of the probes. In order for the terminal phosphate or the phosphate diester groups to bind to the zirconated surface, they must displace the oxide and hydroxide ligands. The terminal phosphate, $ROPO_3^{2-}$, is a stronger base than the backbone $(RO)_2PO_2^-$ groups. In addition, the resulting zirconium phosphate/phosphonate bilayer is structurally very similar to the layers of the solid-state zirconium phosphates (Figure 4), so the process is helped by the stabilizing lattice energy associated with the resulting network structure.

Commonly, a linker is used to separate the support surface from the section of oligonucleotide that is to be used in hybridization.[10,35,38,39,63] The purpose is to increase the availability of the probe to incoming molecules so that the hybridization more closely mimics conditions in solution. Aliphatic segments, ethylene glycol oligomers, or simply a sequence of thymine (T) bases have been used as tethers.[6] While exploring possible tethers for the phosphate-terminated probes, we observed that the nature of the tether can be important. Specifically, we have seen that short segments of guanine (G) oligomer lead to an increase in the fluorescence after hybridization by a factor of two relative to the cases in which no spacer is present (Figure 9). In contrast, a similar polyA spacer has essentially no effect, while polyT and polyC spacers actually lead to decreased hybridization relative to probes with no spacer. These observations are independent of the identity of the probe or its concentration, and were most pronounced for spacers $[(G)_n]$ with $n = 7$–9.

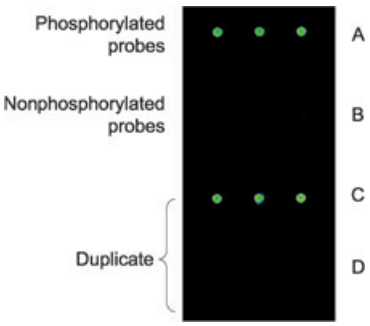

Figure 8. Fluorescence map comparing modified and unmodified probes on zirconium phosphonate slides after BSA treatment and hybridized with the corresponding Cy3-labeled complement. The slides were spotted with 5′H_2O_3PO-$(G)_{11}$-O33(Z) (lines A, C) and O33(Z) (lines B, D) and hybridized with 100 nM complements comp-5′CY3-O33(Z), in which O33(Z) corresponds to a 33 mer oligonucleotide. Spot size is 100 μm. The fluorescence intensities are color coded varying from blue (low) to green, yellow, red, and then white (saturation).

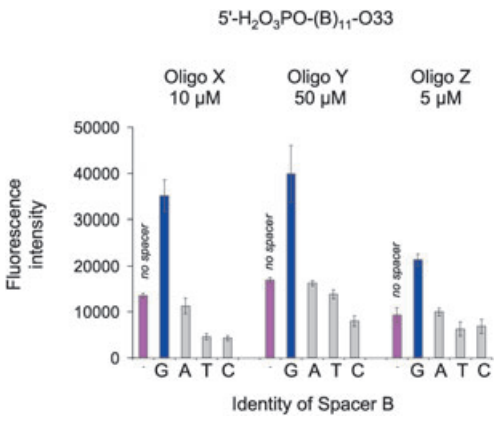

Figure 9. Fluorescence enhancement upon hybridization with labeled target related to the introduction of a polyG spacer to the probe. The effect is present for different probes spotted at different concentrations.

Unlike other homopolymers, polyG does not normally exist in a single-stranded form, and this feature may be responsible for the observed behavior. Studies have shown that single strands associate in parallel or in a variety of antiparallel orientations.[64–70] In the presence of potassium or sodium ions, like in the sodium citrate buffer, strands of polyG can form stable four-stranded helices (Figure 10).[70] A possible consequence of this tendency of polyG segments to associate is an increase in surface coverage of the probes within a spot,

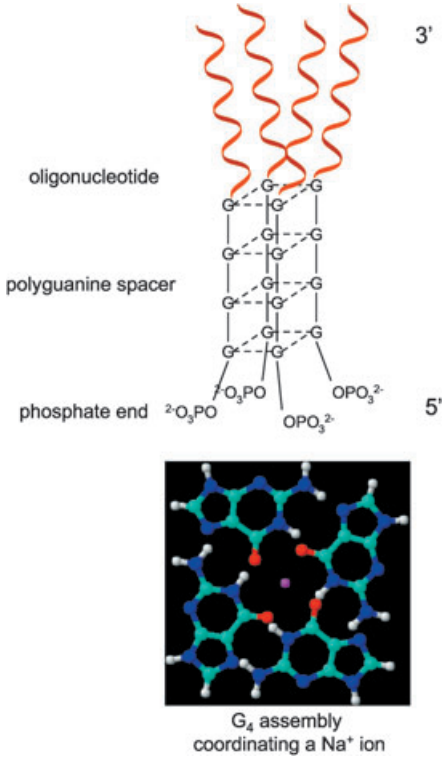

Figure 10. Possible tetraplex formation from polyG aggregates.

either by inducing the probes to pack tighter, or by forming multidentate aggregates that raise the avidity for the surface relative to single probes. Alternatively, the aggregates may be responsible for increased hybridization efficiency by providing rigid tethers that better orient the oligonucleotide for access by the targets.

Double-Stranded Probes

Arrays of ds-DNA probes for the high-throughput screening of sequence-specific DNA/protein interactions involved in gene transcription control, DNA recombination, restriction, and replication are also of interest. Recent illustrations of this powerful approach have been obtained using activated glass slides and 5′-amino-modified ds-DNAs.[71–73] However, commonly used supports such as aminosilane or epoxide-coated slides retain the 5′-amine-modified and nonmodified ds-DNA equally well, because of electrostatic interactions with the DNA phosphate backbone.[74] As with arrays based on single-strand probes, specific binding of the double-stranded probe terminus is preferred to improve the accessibility of the protein binding site to targets.

Table 2. Double stranded DNA binding.[a]

5′ end modification[b]	Sequence X (33 mer) intensity[c]	Sequence X (33 mer) intensity[d]	Sequence Y (33 mer) intensity[c]	Sequence Y (33 mer) intensity[d]
phosphate	8200 ± 1700	1900 ± 600	8300 ± 2900	500 ± 400
$(G)_9$-phosphate	25 5000 ± 3900	9000 ± 1400	25 400 ± 6200	7100 ± 1500
none	1200 ± 150	400 ± 100	1800 ± 300	600 ± 100

[a] Concentration of the spotting solutions: 20 μM in SSC 1X (pH 6). [b] The complementary strand is CY3-labeled in the 5′-position. [c] No BSA rinsing. [d] With BSA rinsing.

Recent experiments with the zirconium phosphonate coated slides also show highly specific adsorption of the 5′-phosphate-modified DNA duplexes (Table 2). A phosphorylated ds-DNA probe, containing a specific sequence recognized by a protein involved in the regulation of gene expression, was spotted on the zirconium phosphonate slides and its ability to capture the corresponding protein was demonstrated (Figure 11). Importantly, phosphorylation at the 5′-

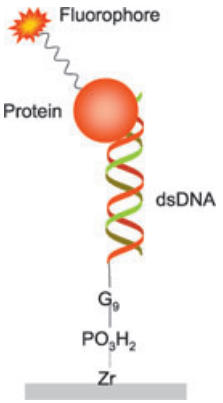

Figure 11. Analysis of protein-(ds-DNA) interactions on zirconium phosphonate slides.

position of the DNA duplexes can be easily achieved either by annealing the target ss-DNA with a universal primer that bears a 5′-phosphate group[73] or by phosphorylation of ds-DNA with a kinase, then avoiding the use of chemically modified nucleotides. A full study is currently underway to confirm the potential of this approach for the analysis of protein-(ds-DNA) interactions.

Outlook and Further Consideration

The results described in this Concepts article illustrate that the mixed organic/inorganic metal phosphonate thin films

represent a viable new approach to DNA-based bioarrays. It is easy to imagine that the idea of interfacing mixed organic/inorganic interfaces can be extended to other applications.[75] For example, the metal–phosphonate-derivatized surfaces should not be limited to DNA arrays. Other biological probes that can easily be modified by phosphate or phosphonic acid groups could also be spotted on the support. Possibilities include arrays based on protein nucleic acids, non-ionic DNA analogues capable of sequence-specific binding to DNA or RNA,[76] or carbohydrates for the evaluation of protein binding. Similarly, the properties of the inorganic surface itself can be tuned to meet specific applications. The zirconium ions used in the work described here can be replaced to change the hard/soft character of the surface to alter the specificity of probe binding.

The concept of using the coordinating abilities of phosphate or related phosphonic acid groups towards metal ions on inorganic surfaces can also be extended to other biotechnology problems. One example is the design of better drug delivery systems that could reduce side effects, improve efficacy of existing drugs, and open the door to entire classes of new treatments. Given that the strength of the interaction between the phosphate or phosphonate and the metal support can be tuned by changing the nature of the metal center, the immobilization of therapeutic agents that naturally bear such functional groups (examples include fosfomycine, foscarnet, and tenofovir) onto inorganic biocompatible carriers could offer great potential in the field of medical devices. In this context, calcium phosphate ceramics (CaPs), commonly used as implants for bone reconstruction[77] appear to be good candidates, since they can be resorbed by bone cells. For example, we were able to chemically combine CaPs with a geminal bisphosphonate (Zoledronate)[78] that is efficient for the treatment of post-menopausal osteoporosis[79] and bone metastases (Figure 12). The ability of the resulting biomaterials to release the bisphosphonate drug was demonstrated by using in vitro[78] and in vivo studies.[80]

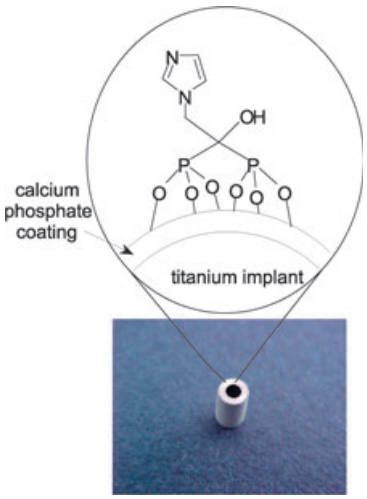

Figure 12. Bisphosphonate coating of calcium phosphate-based orthopedic implants increases peri-implant bone quality in osteoporotic rats.

Acknowledgements

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