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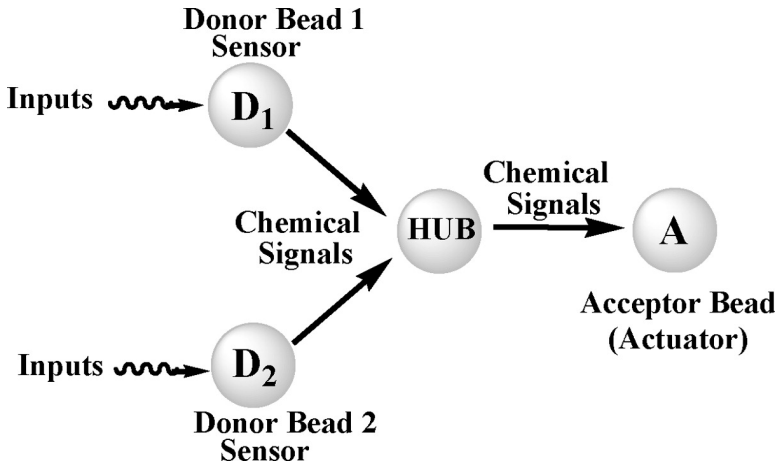

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Networking Particles over Distance Using Oligonucleotide-Based Devices

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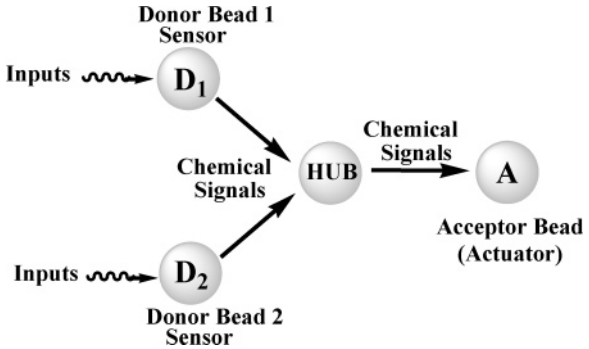
Abstract: Microparticles covered with DNA-based computing elements that sense inputs and release oligonucleotides as outputs could be used to construct autonomous networks with increasingly complex functions. We demonstrate cascades of particles with up to three layers and a nonlinear network with an **AND** gate hub. In order to establish functional networks, particles do not have to be in direct physical contact.

Introduction

The integration of molecular computing devices with a human body for the comprehensive monitoring of health and correction of disease states will require programmable, biocompatible, and self-organizing elements that can be designed to interface with each other and with human tissues. Deoxyribonucleic acids (DNA) provide us with multiple substrates for general silicomimetic computing[1] and for sensing,[2] which can be interfaced with various in vivo biomarker inputs (Scheme 1). Downstream of such inputs, DNA computing can also be used to control elements that perform corrective action, such as drug release or protein activation.[3] In the recent years, solution-phase DNA computing devices of substantial complexity have been designed;[1c,e,4] however, most of these cannot be directly applied in therapeutic situations. Incorporating DNA computing with more traditional approaches to nanomedicine,[5] such as those using nanoparticles or lyposomes as vehicles for drug delivery, would open new possibilities to increase the functional complexity of delivery systems. Herein, we describe an approach that uses DNA devices to establish networks of microscopic particles (Figure 1) in order to coordinate their activity *without any direct physical contact*.[6]

Scheme 1. Principle behind Networks of Microparticles (or Beads)[a]



[a] Donor or sensors beads ($\mathbf{D_1}$ and $\mathbf{D_2}$) sense stimuli (chemical or physical), release chemical signals (here in the form of oligonucleotides), which diffuse to a hub bead that analyzes them. If set of conditions is satisfied, hub releases an oligonucleotide signal to the final acceptor bead or actuator (**A**).

Results and Discussion

The elementary unit of a network of microparticles (in this work, microspheres or beads for flow cytometry) is a single bead covered with a DNA computing or sensing element. Individual bead senses the presence of an input stimulus (or multiple stimuli) in solution, and according to a set of rules encoded on this bead by computing elements, it releases an oligonucleotide signal as an output through a catalytic process (Figure 1). This signal can interact with another DNA element on a downstream bead, and we say that this process leads to the information transfer between beads. We call the first

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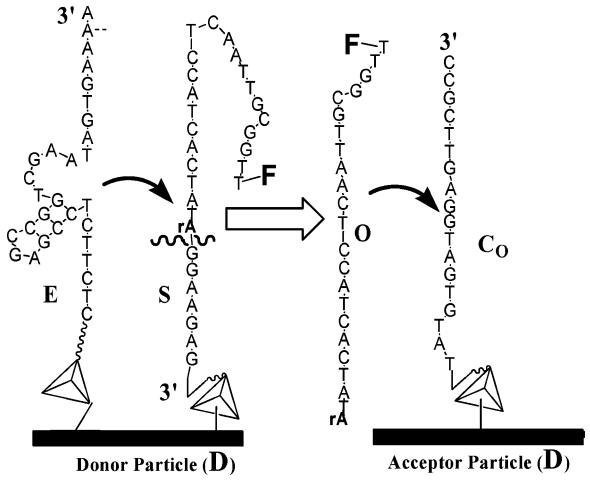
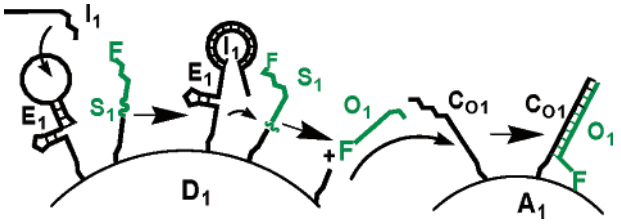


Figure 1. An example of the basic chemistry of communication between two microparticles over distance (donor−acceptor cascade): A donor particle (**D**) coated by nucleic acid enzymes (**E**) and substrates (**S**) can release the product of catalytic cleavage, or output (**O**) labeled with fluorescein (**F**). The released product is captured by a complementary oligonucleotide ($\mathbf{C_O}$) on the acceptor $\mathbf{A_1}$ bead. Initial release can be triggered by any method that can control the activity of nucleic acid enzymes.

Scheme 2. Communication between Two Particles over Distance (Donor−Acceptor Cascade)[a]



[a] Donor bead ($\mathbf{D_1}$) senses a stimuli ($\mathbf{I_1}$), releasing oligonucleotide signals $\mathbf{O_1}$. This is accomplished by coating the bead with a catalytic nucleic acid enzyme $\mathbf{E_1}$ sensitive to the input and substrate $\mathbf{S_1}$. Next, released $\mathbf{O_1}$ is captured by an $\mathbf{A_1}$ bead covered with its complement $\mathbf{C_{O1}}$.

(upstream) bead donor (**D**) and the second (downstream) bead acceptor (**A**). The particle-based approaches have a potential to be "plug-and-play" because networks can be constructed from individual modules by matching the outputs of donor elements to the input of acceptor elements. The communication between elements requires no physical contact between **D** and **A**; that is, it occurs over the long range through diffusion of signaling molecules.

Some of the DNA elements that we tested on beads and that we are presenting in this work include (1) deoxyribozyme-based logic gates,[1a] including catalytic molecular beacons (e.g., Scheme 2, $\mathbf{D_1}$), which upon activation by the input cleave proximal substrates and release the output products; (2) strand displacement elements[1d,7] in which an input displaces an inhibitory oligonucleotide and starts a catalytic reaction that generates an output; this approach has been used extensively in Winfree−Seelig solution-phase circuits;[1d] and (3) structure switching aptamers,[2b] which release oligonucleotides upon sensing analytes[8] (shown in Supporting Information).

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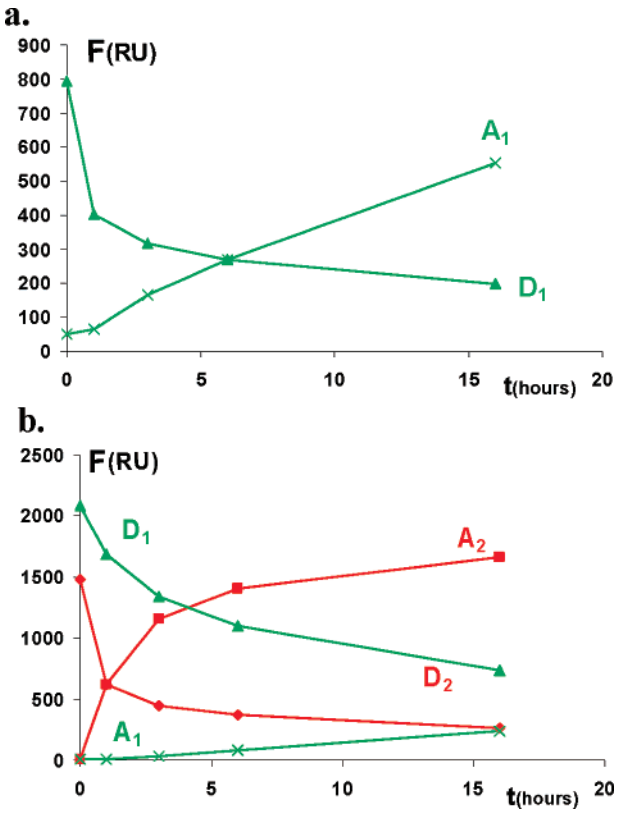
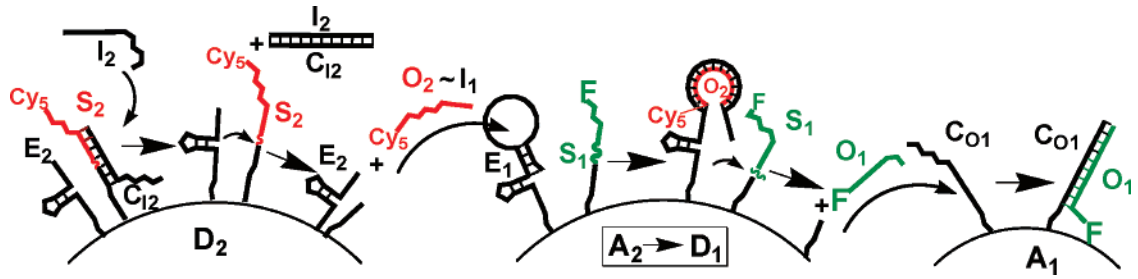


Figure 2. (a) Time course of an information transfer in the presence of an input oligonucleotide $\mathbf{I_1}$ between $\mathbf{D_1}$ and $\mathbf{A_1}$ beads monitored by flow cytometry (fluorescein channel, 488 nm laser excitation, 535 nm filter). Beads were separated by size. (b) Time course of an information transfer between three beads (from $\mathbf{D_2}$ to $\mathbf{A_2}$ which turns into $\mathbf{D_1}$, then from $\mathbf{D_1}$ to $\mathbf{A_1}$) in the presence of an input oligonucleotide $\mathbf{I_2}$; beads monitored by flow cytometry ($\mathbf{D_2}$ to $\mathbf{A_2}$ Cy5 channel, 635 nm laser excitation, 675 nm filter, $\mathbf{D_1}$ to $\mathbf{A_1}$ fluorescein channel, 488 nm laser excitation, 535 nm filter). Beads were separated by size as well. In both graphs, the *y*-axis shows relative fluorescence, and no conclusion can be made based on these intensities, by comparing different beads.

Demonstration of a Communication between Two Particles: We start first with an elementary system with two beads, one donor bead releasing an oligonucleotide upon sensing an input and another acceptor bead capturing the released oligonucleotide. We compare the two situations: (1) both beads are spread in a single layer in a Petri dish, but are mixed together; (2) beads are separated by a polycarbonate membrane (0.4 μm, using Transwell plates). The former situation allows rapid diffusion of information between beads, while the latter is a slower process, but is an important control that beads do not transfer information through a direct contact.

The particles in this work are streptavidin-coated polystyrene or latex beads for flow cytometry. Flow cytometry provides us with convenient means of following the process of information transfer between beads, as beads within networks can be readily differentiated, for example, by their size. In our first cascade (Scheme 2), the donor bead $\mathbf{D_1}$ is a 5.8 μm polystyrene magnetic bead coated homogeneously with deoxyribozyme sensor gate $\mathbf{E_1}$ and its substrate $\mathbf{S_1}$. In the presence of activating input $\mathbf{I_1}$ the enzyme is "turned on", cleaving the substrate and releasing one of the products from the bead in solution as an output ($\mathbf{O_1}$). The product diffuses away from the bead and is captured by the acceptor bead $\mathbf{A_1}$ (8.2 μm polystyrene magnetic microsphere), which is coated with a complement of $\mathbf{O_1}$ ($\mathbf{C_{O1}}$). If the substrate on $\mathbf{D_1}$ is labeled with fluorescein ($\lambda_{exc} = 480$ nm, λ_{em}

Scheme 3. Three-Layer Cascade[a]



[a] The cascade starts with a donor $\mathbf{D_2}$, which senses oligonucleotide $\mathbf{I_2}$. $\mathbf{D_2}$ is coated with a nucleic acid enzyme $\mathbf{E_2}$ and its substrate $\mathbf{S_2}$. Substrate is blocked by a complement to the input ($\mathbf{C_{O2}}$), thus only upon the addition of the input the enzyme can cleave its substrate. The product $\mathbf{O_2}$ is released, behaving as $\mathbf{I_1}$ and activating the sensor gate (catalytic molecular beacon) $\mathbf{E_1}$ on $\mathbf{A_2}$ bead, which is essentially the same as the $\mathbf{D_1}$ bead from Scheme 1. Activated $\mathbf{E_1}$ cleaves $\mathbf{S_1}$ and releases $\mathbf{O_1}$, which is accepted by $\mathbf{A_1}$. $\mathbf{O_2/I_1}$ is labeled with Cy5, while $\mathbf{O_1}$ is labeled with fluorescein, allowing us to observe information transfer down the cascade.

= 525 nm), the whole process of information transfer can be monitored by flow cytometry, typically on thousands of beads at each measurement. The donor strongly signals to the acceptor bead that there is an input in solution within 1 h when beads are just mixed in a single layer in solution (Figure 2a).

In the second experiment, beads are separated by a membrane permeable to oligonucleotides, but not to microparticles, and the measured distance between beads in these experiments was ~4−7 mm. Without stirring or directed flow, the rate of information transfer between the two beads is limited by diffusion, necessitating incubation of at least 6 h (Supporting Information) for a measurable signal on the acceptor bead. We estimate, based on calibration of fluorescence and manufacturers characterization of bead capacities, that in the separated system the donor beads release on average 20−30% of oligonucleotide outputs and that 50−70% of the signal reaches acceptor beads under the conditions described here, in spite of the large volume of our system and the large distance that the chemical signals have to travel. We also note that all our cascades were not optimized to monitor only the last bead in the cascade, but rather to monitor changes in fluorescence for both beads with flow cytometry. Also, we had to decrease the density of fluorescein-labeled substrates in order to avoid the self-quenching and saturation of fluorescence (which would make early phases of signal release difficult to monitor), while at the same time we had to sacrifice potential for catalytic amplification (i.e., used excess of enzyme over substrate).

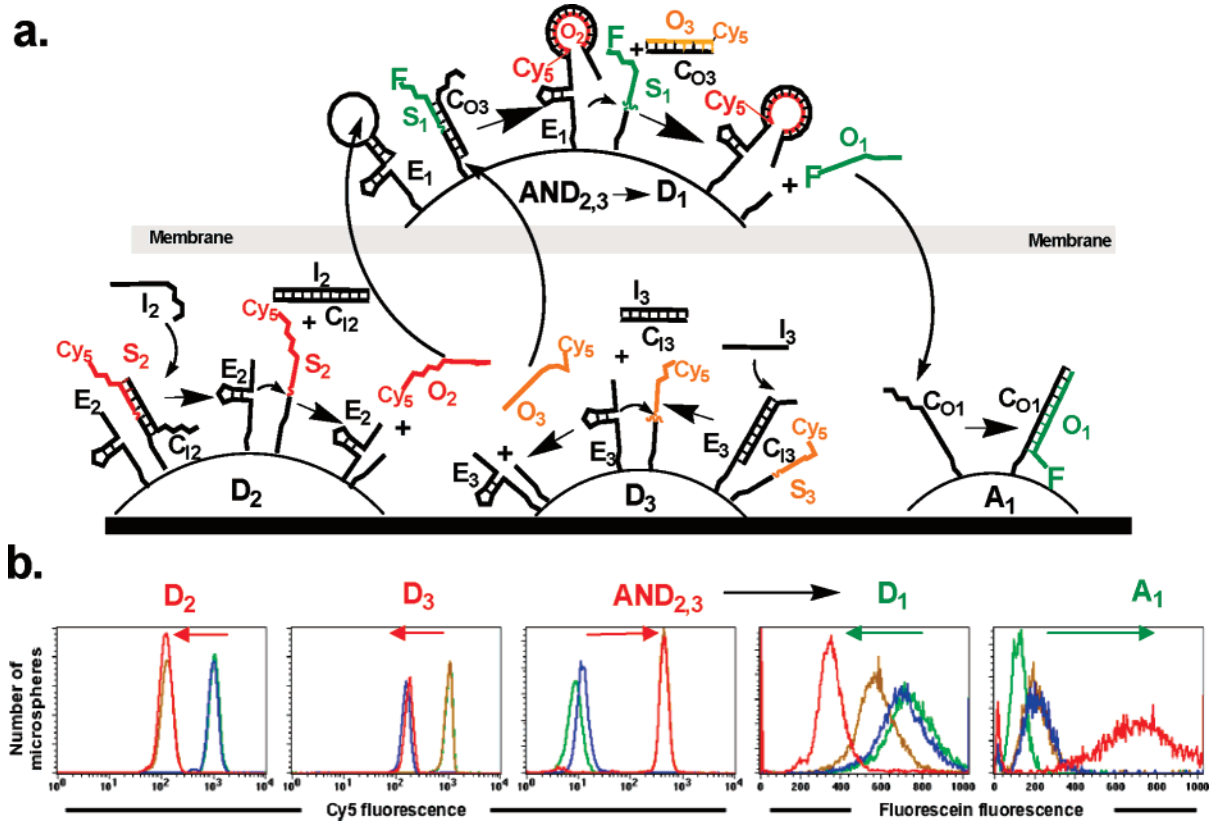
Demonstration of a Three-Layer Cascade: We next added one more component to our system, forming a three layer cascade. Instead of the input $\mathbf{I_1}$ being manually added, as in the simple donor−acceptor system, herein we used upstream beads, $\mathbf{D_2}$ (2.8 μm polystyrene magnetic microsphere), which release $\mathbf{I_1}$ (in Scheme 3 $\mathbf{I_1}$ is renamed $\mathbf{O_2}$) upon sensing the presence of an input oligonucleotide $\mathbf{I_2}$. In order to monitor information transfer between all beds simultaneously by flow cytometry, the substrate on $\mathbf{D_2}$ was labeled with Cy5 dye (λ_{exc} = 648 nm, λ_{em} = 688 nm). We also say that in the three-layer cascade the middle bead transitions from an acceptor to donor state (i.e., $\mathbf{A_2 \rightarrow D_2}$).

The whole system is described in Scheme 3 and can be implemented as a straightforward extension of the two-layer cascade. The new $\mathbf{D_2}$ particles were coated with another enzyme−substrate pair $\mathbf{E_2}$ (same enzyme can be actually used because it is immobilized) and $\mathbf{S_2}$. In order to demonstrate the versatility of elements, we incorporated strand-displacement-based sensing of input $\mathbf{I_2}$ in $\mathbf{D_2}$: an oligonucleotide $\mathbf{C_{I2}}$ complementary to the input was blocking the substrate, and the addition of $\mathbf{I_2}$ would remove the blocking strand, allowing the enzyme to cleave its substrate. It took more time to detect information transfer over the three-layer cascade, and only minor signal increase was observed within the first hour (Figure 2b). We also tested a control cascade in which the first and the last layers were on one side of the membrane, while the middle layer was on the other (Supporting Information). This system worked equally well, except the time needed to observe the strong signal on the last bead in this cascade was 16 h (statistically significant signal was observed already after 3 h). Omitting the middle layer produced no signal in the last layer, demonstrating that this is a true cascade.

Demonstration of an AND Hub: The key step toward building complex networks beyond simple cascades is the creation of "hub" microparticles that would serve to integrate inputs from two or more donor particles into one output. The hubs would allow us to perform the autonomous decision-making with networks of microparticles, with hubs evaluating individual elementary Boolean logical operations, such as **OR** and **AND**, with **NOT** logic being implemented most easily at the first layer of cascades.[1d]

We offer herein a typical example of this approach to the decision-making networks of microparticles: a three-layer network consisting of four types of microspheres, with a hub performing an **AND** logical operation (Scheme 4). We built this system from our three-layer cascade, with the following modifications: We first added an element, a bead $\mathbf{D_3}$ (5.6 μm latex microsphere, nonmagnetic) that sensed a new input, oligonucleotide $\mathbf{I_3}$. On this bead, the enzyme $\mathbf{E_3}$ is blocked with an inhibitory oligonucleotide complementary to $\mathbf{C_{I3}}$. Upon sensing $\mathbf{I_3}$, the enzyme on this bead is activated, thereupon cleaving $\mathbf{S_3}$ and releasing $\mathbf{O_3}$. In order to construct the hub, we next modified the $\mathbf{A_2}$ bead (which was in turn obtained by modifying $\mathbf{D_1}$ in Scheme 2): we blocked the extended substrate $\mathbf{S_2}$ by an oligonucleotide $\mathbf{C_{O3}}$, which was removable by the addition of $\mathbf{O_3}$. In our design, the hub would active if (1) $\mathbf{O_3}$ would remove $\mathbf{C_{O3}}$ and, independently, (2) $\mathbf{O_2}$ would activate the enzyme. Thus, the whole bead is actually behaving as an $\mathbf{AND_{2,3}}$ gate. Because the first layer of beads is active only when inputs $\mathbf{I_2}$ and $\mathbf{I_3}$ are present, we say also that the whole network behaves as an **AND** gate, with these oligonucleotides as inputs and the increase in fluorescence at $\mathbf{A_1}$ bead as an output. In order to facilitate the monitoring of this system with flow

Scheme 4. The Schematic Representation of an **AND** Network Made of Four Microparticles, with a Central Bead Acting as an **AND** Hub[a]



[a] (a) Beads $\mathbf{D_2}$ and $\mathbf{D_3}$ report the presence of oligonucleotides $\mathbf{I_2}$ and $\mathbf{I_3}$, respectively, to the hub bead $\mathbf{AND_{2,3}}$, by releasing $\mathbf{O_2}$ and $\mathbf{O_3}$. $\mathbf{O_2}$ and $\mathbf{O_3}$ activate elements on the hub, which is transformed into $\mathbf{D_1}$ ($\mathbf{AND_{2,3}}\rightarrow\mathbf{D_1}$), releasing output $\mathbf{O_1}$, which is in turn captured by $\mathbf{A_1}$. Individual elements are as described in Schemes 2 and 3, except for $\mathbf{D_3}$. (b) The flow cytometry results presented as panels with numbers of microspheres with certain fluorescent intensity (relative units); each panel is for one of the components of the network (hub with two panels because it both receives and releases oligonucleotides), with four experiments shown in each panel (both inputs, individual inputs, and no inputs). Color coding of experiments: red, both $\mathbf{I_2}$ and $\mathbf{I_3}$ added; brown, $\mathbf{I_2}$ added; blue, $\mathbf{I_3}$ added; green, neither added. $\mathbf{D_2}$ and $\mathbf{D_3}$ panels show decrease of fluorescence due to the release of Cy5-labeled $\mathbf{O_2}$ and $\mathbf{O_3}$, respectively; $\mathbf{AND_{2,3}}$ panel shows an increase in fluorescence in Cy5 channel due to capturing of $\mathbf{I_2}$ ($\mathbf{I_3}$ is not captured by the bead); $\rightarrow\mathbf{D_1}$ panel shows decrease in fluorescence due to release of fluorescein-labeled $\mathbf{O_1}$; $\mathbf{A_1}$ panel shows an increase in fluorescence upon binding of $\mathbf{O_1}$.

cytometry, four beads were immediately tested in an experiment using membrane to separate the hub from the other bead, analyzing it separately. We needed again prolonged incubation to observe the strong signal. Although there was a clear digital behavior at the $\mathbf{A_1}$ bead (Scheme 4b, $\mathbf{A_1}$ panel), with 4-fold increase in fluorescence, we also note some imperfect digital behavior in the hub, particularly, in the presence of only $\mathbf{I_2}$. The dissipation of the small signal in the large volume of the reaction, however, led to the suppression of the imperfect digital behavior in the final layer (akin to noise suppression, or threshold). A similar network with small molecules (e.g., ATP or steroid) as one of the inputs with a structure switching aptamer used as a DNA element and an oligonucleotide as another input is also reported in our Supporting Information.

Conclusion

The silicomimetic DNA computing and sensing (e.g., based on aptamers, cf., Supporting Information) can be adapted to accomplish the integration of microparticles into cascades and networks, and these networks are capable of more complex functions than individual particles. The dynamic networks of beads are of interest not only as a new computing medium but also for their potential in nanomedicine, for example, in molecular computing-controlled drug release. This approach can be used, we hope, to minimize the side effects of targeted immunotherapy whenever nanoparticles are used. Our results represent a step toward a scenario in which networks will be used to assess the presence of multiple tissue markers, or they could assess additional information from the remote positions. In the current nanomedicine paradigm, more complex functions of microparticles are achieved by loading a single particle with additional functionalities. We now demonstrate a nonlinear approach, in which an increase in complexity of functions is achieved by forming networks of simple(r) particles.

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Supporting Information Available: All sequences and experimental procedures, including results for (1) through-membrane communication for Schemes 2 and 3, (2) two-particle ATP donor−acceptor chain, (3) four-particle ATP-oligonucleotide with hub AND bead. This material is available free of charge via the Internet at http://pubs.acs.org.

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