Aptamers Embedded in Polyacrylamide Nanoparticles: A Tool for *in Vivo* Metabolite Sensing

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ABSTRACT We describe a new type of aptamer-based optical nanosensor which uses the embedding of target responsive oligonucleotides in porous polyacrylamide nanoparticles to eliminate nuclease instability. The latter is a common problem in the use of aptamer sensors in biological environments. These aptamers embedded in nanoparticles (AptaNPs) are proposed as a tool in real-time metabolite measurements in living cells. The AptaNPs comprise 30 nm polyacrylamide nanoparticles, prepared by inverse microemulsion polymerization, which contain water-soluble aptamer switch probes (ASPs) trapped in the porous matrix of the nanoparticles. The matrix acts as a molecular fence allowing rapid diffusion of small metabolites into the particles to interact with the aptamer molecules, but at the same time it retains the larger aptamer molecules inside the nanoparticles providing protection against intracellular degradation. We tested the ability of the AptaNPs to measure the adenine-nucleotide content in yeast cells. Our results successfully demonstrate the potential for monitoring any metabolite of interest in living cells by selecting specific aptamers and embedding them in nanoparticles.

KEYWORDS: nanoparticles · aptamers · metabolite monitoring · *Saccharomyces cerevisiae* · nanosensor · PEBBLE · molecular beacon

Metabolite sensing is an important tool in understanding cell metabolism. However, with current technologies it is only possible to measure a few metabolites *in vivo* without the need to first quench the cells and subsequently extract the cell material for later analysis. In recent years there has been an interest in using optical nanosensors to monitor *in vivo* metabolite dynamics of a number of cell intermediates. Probes encapsulated by biologically localized embedding (PEBBLE) defines a class of nanosensors designed for minimally invasive analyte monitoring *in vivo*.[1] Nanoparticles possess unique properties to develop better optical sensing mainly because of their large surface areas achieved by small size.

Nanosensors are chemically stable nanoparticles loaded with a sensing component. The sensing involves permeation of a small molecule target into the particle matrix where it interacts selectively with the sensing element leading to a measurable signal change. Polymeric nanosensor systems have been based on fluorescent indicator dyes or encapsulated target-specific biological receptors such as DNA, proteins, or peptides as reviewed recently.[2] For example, a phosphate nanosensor was developed by embedding fluorescent reporter proteins responsive to phosphate ions in polyacrylamide nanoparticles. In another example, horseradish peroxidase embedded in polyacrylamide nanoparticles was designed to measure reactive oxygen species (ROS).[3]

All the previously reported nanosensors were designed for the target of interest by finding dyes or receptor molecules that interact specifically with the analyte of interest and this limits the development of new nanosensors. However, the embedding of aptamers might open up a new class of sensors that are more widely applicable, since it is possible to artificially select an aptamer for any metabolite of interest, and therefore aptamer-based PEBBLE nanosensors can be a generic system for *in vivo* monitoring of small molecules.

Aptamers are single-stranded oligonucleotides that can bind to specific targets. Nucleic acid aptamers may comprise unmodified DNA and RNA as well as chemically modified forms. They have similar properties to antibodies in that they bind targets with high affinity and specificity. Furthermore, they possess additional properties making them superior to antibodies: They are easy to synthesize chemically and they can be developed by artificial selection. Therefore, they are considered to be powerful candidates for developing better sensors for diagnostic or therapeutic applications. Aptamers are selected from a combinatorial library through an artificial evolution procedure called SELEX[4,5] and have

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been used in many formats of biosensing since then.[6] There has been an exponentially growing number of applications of aptamers as biosensors for the last two decades as reviewed in several recent publications.[7,8] One of the main attractive properties of aptamers is the *in vitro* selection procedure which can provide an aptamer against virtually any target of choice and with a predefined affinity. Examples of selected aptamers in the literature back up this claim as aptamers have been reported for a wide range of targets, including whole cells, proteins, and small molecules like ATP. Moreover, nucleic acids have unique properties that can be exploited in biorecognition applications. Aptamers are often produced by chemical synthesis, and hence functional molecules like fluorophores can readily be conjugated to aptamer molecules during synthesis. Moreover, the structural flexibility of oligonucleotides allows for adaptation of the aptamer to various structure changing formats like molecular beacons.[9,10] Aptamer beacon designs allow for conversion of a selected aptamer into an optical reporting molecule for a specific ligand. Aptamer beacons are rationally designed sensors so that an interaction with a target leads to a separation of a quencher and a fluorophore at two distant ends of the aptamer molecule and signals the presence of the target molecule.

Aptamers have been used for *in vivo* targeting, for sensing, and for therapeutic purposes. A DNA oligonucleotide assembly has been used to measure cytoplasmic pH.[11] However, it is well-known that *in vivo* environments limit the direct use of molecular beacon-type probes because of endogenous nucleases and single strand DNA-binding proteins which are causing false positive signals.[12] Aptamers, especially in a living environment, are susceptible to endo- and exonucleases. Furthermore, RNA oligos are degraded rapidly *in vitro* as well as *in vivo*. Although DNA oligos are relatively resistant to nucleases *in vitro*, they are degraded quickly *in vivo*. As stability-enhancing strategies, choosing the positions for modifications on selected aptamers by molecular modeling or selecting new aptamers from modified oligonucleotide libraries have been used to increase nuclease stability of aptamers to some extent. Such modifications on the base, backbone, or sugar moieties of oligonucleotides resulted in a reduced level of degradation by nucleases and hence an increase in half-life of oligonucleotides *in vivo*,[13] but sensitivity to degradation of aptamers is still a major concern[14] because of limited success and extra costs involved in the modification procedures.

We report here a nanosensor consisting of an adenine-nucleotide responding aptamer switch probe (ATP-ASP) embedded in polyacrylamide nanoparticles. The nanoparticles (NPs) have a diameter of about 30 nm and were prepared by inverse microemulsion polymerization. Embedding aptamers in nanoparticles combine the desirable properties of nanoparticles with those of aptamers to achieve a stable metabolite sensor which was tested in yeast cells to measure the total cytoplasmic concentration of adenine nucleotides.

RESULTS AND DISCUSSION

Aptamers are embedded in the matrix of polyacrylamide nanoparticles with the aim of using them as nanosensors to measure spatial and temporal dynamics of metabolites in cell cytoplasm. An ATP binding aptamer has been used in this study as a proof-of-concept. The overall strategy is summarized in Figure 1.

To measure the ligand binding events, the aptamer should be engineered with a transduction mechanism in order to obtain a signaling molecule. Numerous forms of transduction designs have been reported in the literature.[8,15] A thrombin aptamer beacon was constructed to detect an aptamer−ligand interaction by using a classical molecular beacon concept originally developed for detection of DNA−DNA interactions.[16] We chose one type of available molecular design which is an improved molecular-beacon-type switch probe responding to adenine nucleotides. The presence of the target is observed by increasing fluorescence as recently described for the ATP-ASP and the Thrombin-binding DNA aptamer switch probe.[17] The switch probes are designed to bring a fluorophore and a quencher molecule at the two ends of an oligonucleotide into close proximity by formation of a hairpin structure. The binding of the target molecule to the aptamer sequence induces an intramolecular structural rearrangement resulting in the separation of the quencher from the fluorophore, thus causing an increase in fluorescence. An unrelated sequence of DNA was converted to a switch probe (control-ASP) in the same way for control experiments. The sequences of the switch probes are illustrated in Figure 2.

Polyacrylamide nanoparticles have previously been reported to have high batch-to-batch reproducibility as demonstrated for nanosensors of pH, calcium, zinc, magnesium, and phosphate.[1,18] The first step in establishing the usefulness of an aptamer-based nanosensor was to synthesize nanoparticles with aptamer switch probes trapped inside. The polyacrylamide particles were synthesized by inverse microemulsion polymerization by mixing aptamer switch probes (ATP-ASP or control-ASP) with acrylamide monomers before the start of polymerization. The resulting particles were characterized by dynamic light scattering (DLS) which showed that the nanoparticles were 30−35 nm in diameter (Supporting Information, Figure S1) and stable for at least 2 days in water or the cytosol buffer used in this study (see the Methods section). The size of the nanoparticles did not seem to be affected by the encapsulation of aptamer molecules as demonstrated by DLS measurement of empty NPs versus AptaNPs. The nanoparticles could retain embedded aptamers inside for at

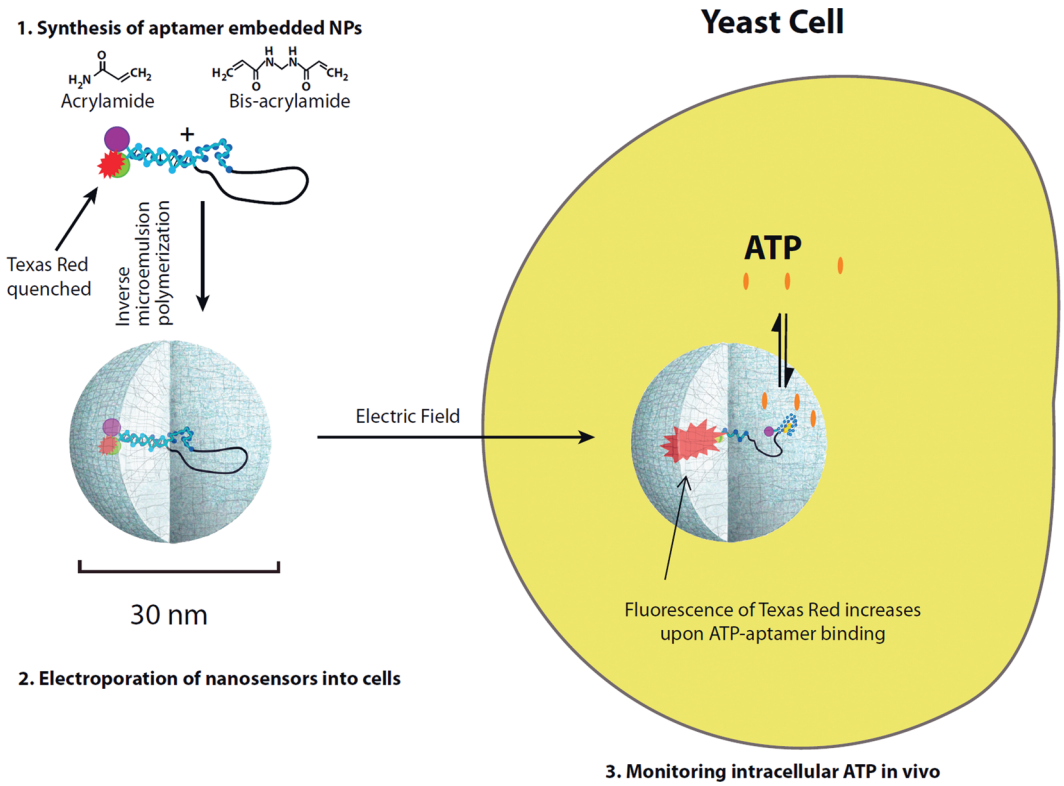

Figure 1. Overall strategy of the use of nanosensors with embedded aptamers in intracellular ATP measurements: (1) aptamers are mixed with acrylamide monomers and a polymerization reaction resulted in 30 nm particles with aptamer probes trapped inside; (2) short electric pulses induce temporary pores in the cell membrane allowing nanosensor particles to be transferred into cell cytoplasm; 3) intracellular metabolites, *e.g.*, ATP, are small enough to pass through pores and bind rapidly and reversibly to the aptamer. Hence, the change in fluorescence of the attached fluorophore represents a change in concentration of the metabolite (ATP).

least 72 h according to a leaching assay (Supporting Information, Figure S2).

Next we tested the ATP-ASP for their ability to bind their target after being encapsulated in the polymer matrix, or if their signaling function was damaged in the radical polymerization process. The behavior of ASP inside the nanoparticles showed a similar response to titration with ATP as the free ASP in solution (Figure 3). Although the response of the ASP used in this study was similar to the previous report in responding to ATP,[17] the signal was significantly reduced. This reduction in signal is most likely due to the use of a different fluorophore-quencher pair (Texas Red−BlackHole2 *versus* Ce6−BlackHole2) and to differences in buffer composition (higher salt concentrations, presence of glucose, and BSA). One consideration in establishing the aptamer-containing nanoparticle as a useful biosensor is the solution's electrolyte composition, because aptamers are known to have an affinity profile dependent on the salt content of the solution as will be discussed further below. All experiments in this study were therefore performed in a specifically designed cytosol buffer (see the Methods section). DNA AptaNPs were used to assess the signaling response to ATP, ADP, and AMP since ATP will be converted to ADP during metabolic reactions and considerable amounts of ATP and ADP exist in cytoplasm. There was no binding to GTP and the aptamer switch probe signaled similar levels of fluorescence increase for all adenine-nucleotides (ADP, AMP, and ATP) (Figure 3), in good agreement with previous reports. The selected unmodified DNA aptamer was reported to be responsive to adenosine, ATP, ADP, and AMP with dissociation constants in the range 6−40 μM.[19,20] In the present study we found a dissociation constant for ATP of 273 μM for both the ATP-ASP and the ATP-NP in cytosol buffer. However, a loss of affinity following postmodification is a general characteristic for optical biosensors based on structural transduction for signaling.[8] For example, the ATP responsive aptamer engineered by conjugating fluorescence reporters was found to have a higher K_d than those of parental aptamers (300 μM *versus* 6 μM).[21] Extensive studies on the DNA ATP aptamer showed that

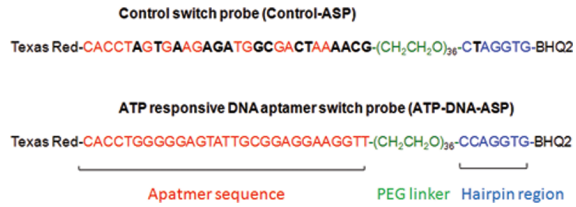

Figure 2. Apatmer switch probe used in this study. Sequences of DNA switch probes and sequences of aptamer regions were selected by Huizenga and Szostak,[19] black colored nucleotides indicate the nucleotides different from the ATP-responsive aptamer switch probe.

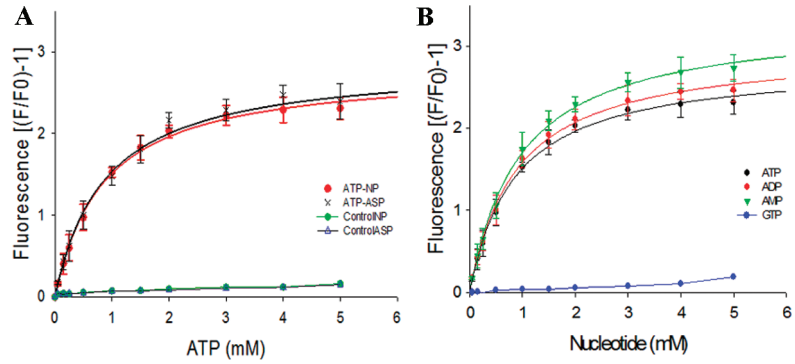

Figure 3. Binding responses of naked aptamer switch probes and probes embedded in NPs. (A) Comparison of the fluorescence response of naked ATP switch probes in solution and embedded inside polyacrylamide NPs as a function of ATP concentration, control-ASP, or controlNP (nanoparticles containing the controlASP do not respond to ATP). Aptamer switch probes signal ATP addition similarly when they are naked or embedded in nanoparticles. (B) Response of AptaNPs to ATP, ADP, AMP, and GTP in cytosol buffer. AptaNP sensors in solution responded identical for all adenine-nucleotides. The binding pattern followed a hyperbolic curve fit. The concentration of naked switch probes was 50 nM and that of AptaNps was 0.5 mg/mL. All experiments were made in cytosol buffer (20 mM phosphate buffer, pH = 7.3, 140 mM KCl, 10 mM NaCl, 2% BSA, 5 mM $MgCl_2$, 5.5% glucose). Fluorescence intensity was normalized by dividing by initial fluorescence intensity (F_0) without ATP and then adjusting to zero.

ligand/aptamer interactions involved both the adenine and the ribose part of target molecules.[22] The minimal functional size of the ATP aptamer was 25 bp. An NMR structural study later showed that the ligand interacts with G8 and also involves a G21:G6 and G18:A10 mismatch.[22] The complex contains two nonequivalent ligand binding sites. The two adenine-nucleotide binding sites are located in the minor groove of a DNA helix. Therefore, aptamer switch probes based on the DNA sequence selected by Huizenga as used in this study will signal the total adenine-nucleotides (ATP, ADP, AMP, and adenosine concentration) in cytoplasm during *in vivo* experiments.

The ATP-ASP was developed to signal aptamer/target binding events. Many other types of designs of fluorescent probes based on aptamer structural changes upon target binding exist, as reviewed recently.[8] The main advantage of the ASP design is the rapid and reversible response to target which is invaluable in applications needed to monitor rapid concentration changes. Kinetic properties of the ASP design are superior to other displacement-type aptasensors available in the literature. For example, bipartite and tripartite aptamer beacons are structure switching sensors based on displacement of a complementary oligosnucleotide which disrupts the aptamer recognition sequence.[23] Kinetic properties of a thrombin-binding aptamer displacement beacon has been evaluated, and the response time was found to be in seconds.[24] The data in Figure 4B confirm the instant response of AptaNPs in a cytosol buffer as reported previously.[17] We determined the response time to be less than 2 s under the experimental setting used in this study.

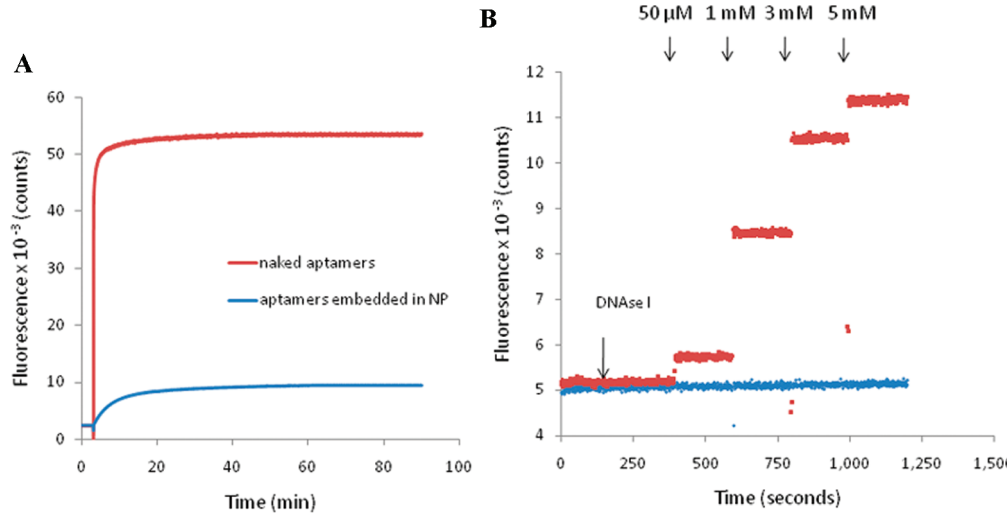

Figure 4. (A) Nuclease stability of DNA−ATP aptamer in solution and embedded in NPs, The concentration of DNase used in assays was 0.5 units/µL and the concentration of AptaNPs was 0.5 mg/mL, the concentration of naked aptamer was 50 nM; (B) AptaNPs (red line) or controlNPs (blue line) were pretreated with nuclease for 90 min, washed with ethanol, and tested for further DNase resistance by adding 0.5 unit/µL at the indicated time. A titration of ATP with the indicated final concentrations signaled similar levels of responses as Figure 3B. All experiments were performed in cytosol buffer.

Structural analysis of the ATP-responsive DNA aptamer showed that the triphosphate was directed out of the binding pocket, resulting in similar affinities for ATP, ADP, or AMP.[22,25] To obtain better selectivity toward ATP, an ATP-responding RNA aptamer was later selected by Sazani *et al.*[26] with the aim of obtaining triphosphate specific aptamers. We have chosen to use the ATP-responsive DNA aptamer in this study because converting RNA aptamers into signaling designs is a challenging task.[15] However, we also tried to convert the ATP-specific RNA aptamer sequence selected by Sazani *et al.*[26] into a molecular beacon design and showed that after embedding it in nanoparticles it responds in a similar way as the DNA AptaNP to increasing concentrations of ATP (Supporting Information, Figure S3).

We then examined the protection of aptamers encapsulated in polyacrylamide nanoparticles against degradation. We hypothesized that embedding aptamer-based probes inside porous nanoparticles would serve two distinct functions: (i) it would prevent degradation of aptamers during delivery of sensors to the site of interest inside the cells, thus keeping the aptamer probes functional, and (ii) it would allow small molecules to diffuse freely in and out of the nanoparticles, thereby permitting a fast and reversible measurement of small molecule concentration in the environment.

Aptamers are susceptible to degradation by nucleases in biological environments. This is especially a problem for RNA aptamers. DNA aptamers are relatively stable *in vitro*, but are rapidly degraded *in vivo*. The half-life of foreign DNA in the cytoplasm of HeLa and COS cells was determined as 50−60 min, and the degradation of DNA was attributed to nuclease attack.[27,28] The ubiquitous presence of RNases results in rapid degradation of RNA aptamers to nucleotides both *in vitro* and *in vivo*. We tested the stability of AptaNPs in degradation assays. DNA aptamers in solutions are degraded over the course of an hour when an external DNase I was added and there was no intact aptamer left to respond to ATP. The aptamers embedded in nanoparticles were resistant to treatment with DNase I. Figure 4A shows that the addition of 0.5 units/μL of DNase I leads to a rapid increase in fluorescence of the DNA−aptamer in the absence of target, but a significantly smaller increase for the corresponding Apta-NPs. The fluorescence increase in AptaNPs by DNase treatment can be attributed to degradation of aptamers that have not been completely embedded in the matrix and thus are subject to partial degradation at the parts of the aptamer molecule that are outside the protective matrix. These observations suggest that nanoparticle embedding protects DNA−ASPs against nucleases and leaves the sensors functional.

As shown in Supporting Information, Figure S3, also, RNA-based aptamers are protected from nuclease attack when encapsulated in polyacrylamide nanoparticles. RNases are ubiquitous in the environment, and the common practice in molecular biology techniques is to include RNase inhibitors in solutions used during the handling of RNA or to use RNase free materials specifically prepared for RNA handling. This phenomenon is a major problem in RNA aptamer applications in biological environments. There have been efforts to prevent such degradation by including modified nucleotides in the aptamer sequences such as phosphorothioate linkages or 2′-protection modifications (2′-*O*-methyl, 2′-fluoro, locked nucleic acids). Chemical modifications had limited success to lower degradation rates of aptamers such that the properties of the aptamer remain relatively unaffected. Although chemical modifications have been reported to be possible for maintaining the original unmodified affinity for a few aptamers, empirical trials are always necessary to verify the function of post-SELEX modified aptamers. Circularization of aptamer molecules, which is achieved by the ligation of two ends of an aptamer molecule, represents another method to gain nuclease-resistance as a post-SELEX modification. Circular aptamers were shown to be relatively resistant to exonuclease degradation compared to linear forms in blood serum.[29] While circularization provides resistance against nuclease degradation by preventing exonuclease activity, the presence of endonucleases is still a concern for *in vivo* aptamer stability. The primary degradation of nucleic acids in serum is through a progressive 3′ exonuclease attack.[30] However, numerous cellular endo- and exonucleases are known to be part of the defense against foreign nucleic acids.[30] Therefore, a protective cover in the form of a nanoparticle would be a better and simpler solution for small target detection as demonstrated here. Nucleases have molecular weights in the range of 30 kD. Most metabolites in cells have molecular weights of less than one kD and are therefore small enough to enter into the porous matrix of the nanoparticles. AptaNP protection is an important criterion for *in vivo* utilization of nanosensors because of the otherwise rapid degradation of foreign oligonucleotides in blood and in cytoplasm as reported for *in vivo* studies.[13,14]

Structural instability of aptamers under different buffer, ionic, and temperature conditions is another consideration. Oligonucleotides can fold into alternative structures under physiological conditions, often resulting in altered binding capabilities. As a rule of thumb, an aptamer retains its original binding properties best in a buffer similar to the selection conditions used during SELEX.[8,31] Here we have tested aptamer response in a solution mimicking the cytoplasm (cytosol buffer) as described in the Methods section. The main difference compared to the original selection buffer[19] was a lower magnesium concentration in the cytosol buffer.

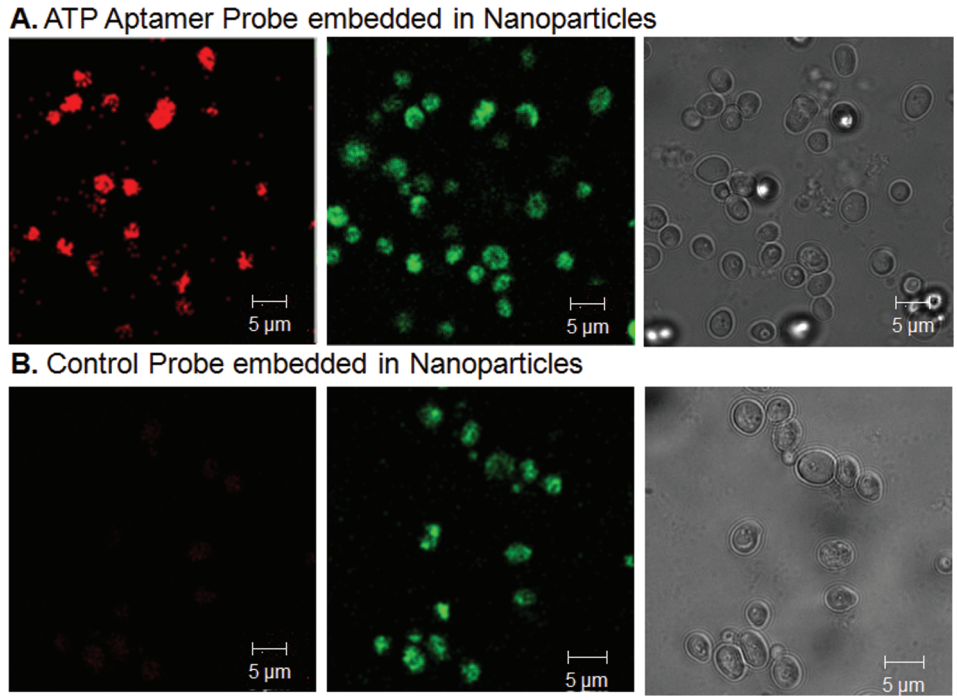

Figure 5. Confocal microscopy images of yeast cells electroporated with (A) ATP aptamer probes embedded in nanoparticles or (B) control probe embedded in nanoparticles. NPs were labeled with a reference dye, Oregon Green (green channel), during synthesis and red fluorescence shows the signaling of aptamer probes interacting with adenine nucleotides inside the cells. An evenly distributed fluorescence after electroporation suggests cytoplasmic delivery of particles. Reference dye intensity shows delivery of particles at similar levels, and the intensity difference between ATP aptamer and control probe indicates that signaling of the aptamer embedded in nanoparticles is functional inside the yeast cells.

After evaluating the AptaNPs for nuclease stability and target response, we tested the performance of the nanosensors for *in vivo* monitoring by inserting AptaNPs into yeast cells. As discussed above, there was a limited amount of degradation by external nucleases when aptamer switch probes were embedded in nanoparticles. However, even a limited degradation can lead to increases in fluorescence and corrupt the determination of the adenine-nucleotide concentration inside cells. Thus, we pretreated all the AptaNPs with DNase to obtain nanoparticles with probes completely resistant to nuclease attack. Figure 4B shows a test for nuclease resistance of pretreated NPs. Addition of more nuclease to a solution of pretreated NPs did not lead to any additional increase in fluorescence both for the control switch probe and for the ATP aptamer switch probe embedded in nanoparticles. This means that pretreatment removed any aptamer which was exposed to the outside of the nanoparticles. However, the aptamers embedded in nanoparticles signal the addition of ATP at similar levels to those in Figure 3A. This indicates that intact switch probes are protected inside the matrix of nanoparticles. The functionality of the controlNPs (control-ASP embedded in nanoparticles) was confirmed using melting temperature analysis (Supporting Information, Figure S4).

The AptaNP sensor was used to visualize and measure the distribution of adenine-nucleotide concentrations in the cytoplasm of yeast cells. For this purpose, a facilitative or active delivery method is required for incorporating nanoparticles into yeast cells.[32] In this study, the nanoparticles were delivered to yeast cells by electroporation.[33] We tested electroporation conditions during nanoparticle delivery by a nuclease assay similar to that shown in Figure 4B to ensure that electroporation experiments did not cause any leaching of switch probes out of nanoparticles: AptaNPs were first subjected to electroporation in solution without yeast cells and the particles were then precipitated and washed. A nuclease assay did not result in any change in fluorescence intensity, showing that switch probes remained inside the particle matrix during the electroporation procedure (data not shown).

Figure 5 panels A and B show confocal microscopy images of yeast cells electroporated with AptaNPs and with controlNPs, respectively. The images of Oregon Green for AptaNP and controlNP show similar levels of delivery of nanoparticles into yeast cells. The even distribution of nanoparticles inside cells suggest a cytoplasmic delivery. The images of cells with inserted aptaNPs showed significantly different Texas Red fluorescence compared to cells with inserted controlNPs. ATP responding aptamers embedded in nanoparticles were mainly in the open state compared to the control probe. We therefore conclude from the images in Figure 5 that the aptaNPs signal adenine nucleotides inside the cells. The signaling levels of cell populations were quantified by using flow cytometry. An approxi-

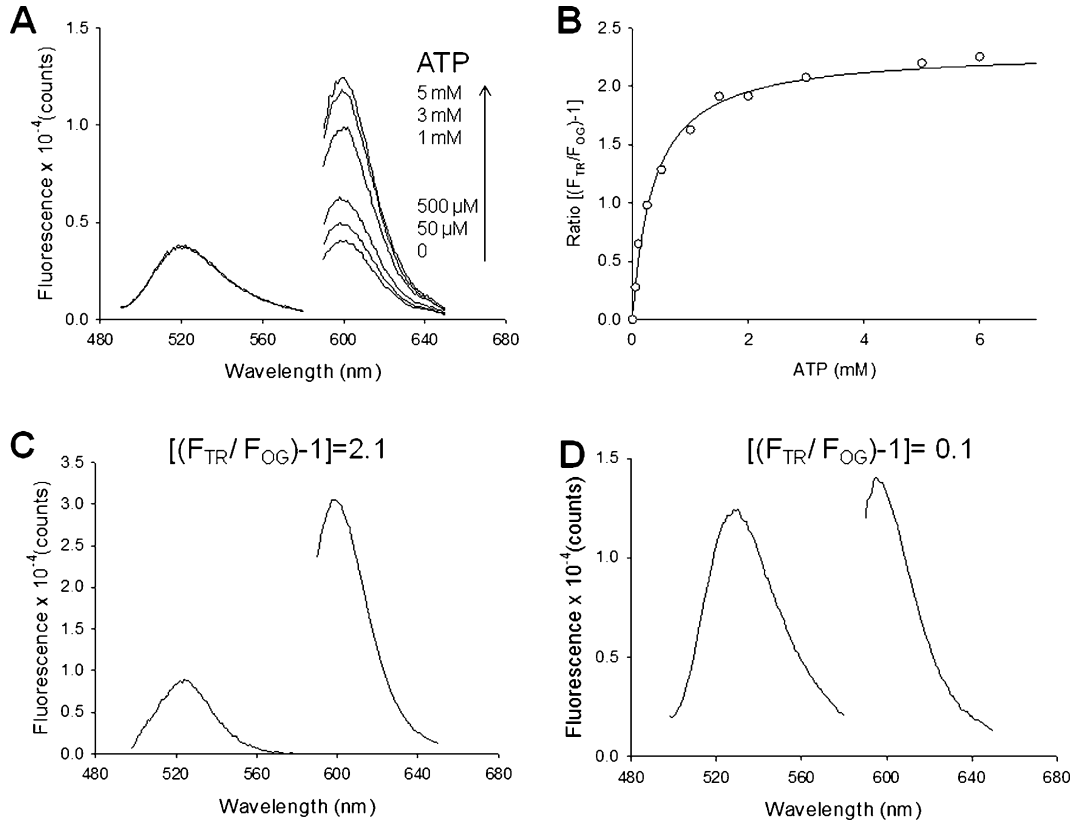

Figure 6. Ratiometric determination of total intracellular concentration of adenine-nucleotides. (A) Emmission spectra (λ_{exc}, Oregon Green = 480 nm, λ_{exec}, Texas Red = 580 nm) and (B) calibration graph of AptaNPs for a titration with ATP in cytosol buffer. The ratio of peak values of fluorescence intensity at 600 and 520 nm was used to construct the calibration graph. (C) To investigate the amount of total adenine-nucleotides in cytoplasm, AptaNPs were electroporated into yeast cells. The emission spectra for Texas Red and Oregon Green were normalized by subtracting those of equal amounts of untreated yeast cells. The ratio of peak intensities at 600 and 520 nm was used to calculate total adenine-nucleotide concentration by using the calibration graph. The average ratio obtained from three independent experiments was 2.1 ± 0.15. (D) Control nanoparticles containing a nonresponsive DNA switch probe were electroporated into yeast cells at similar conditions. The controlNP resulted in a ratio of 1.1 indicating that the control switch probe remained closed in cytoplasm. TR represents Texas Red, and OG is Oregon Green.

mately 1.5 fold of increase in fluorescence of Oregon Green and Texas Red were observed after electroporating the cell populations with AptaNPs (Supporting Information, Figure S5).

The quantification of intracellular fluorescence measurements has well-known inherent drawbacks. Fluorescence lifetime measurements have been used to overcome such problems, but with extra instrumentation.[34,35] Ratiometric detection is another method commonly used in quantitative analysis of fluorescence intensity. The ability of nanoparticles to accommodate multiple dyes allows for a ratiometric determination of target concentration by insertion of a reference dye along with signaling dyes during synthesis of nanosensors. An alternative is a postsynthesis conjugation of reference dye on the surface of the nanoparticles.[36] Both methods of including a reference dye for ratiometric analysis can be applied to polyacrylamide nanoparticles, and such particles were recently used for ratiometric measurements of intracellular pH.[37]

The total concentration of adenine-nucleotides in yeast cell cytoplasm was determined by fluorescence spectroscopy analysis. A ratiometric calibration curve was constructed from *in vitro* titration experiments with ATP (Figure 6A). Oregon Green did not respond to ATP, and the ratio of Texas Red emission to Oregon Green emission followed a hyperbolic curve (Figure 6B). Yeast cells electroporated with AptaNPs or controlNPs were analyzed in order to estimate the adenine-nucleotide concentration (Figure 6C) using the ratiometric calibration graph. The control nanoparticles showed no opening of the control switch probe inside cells as already confirmed by confocal images (Figure 6D and Figure 5B).

The total concentration of cytoplasmic adenine-nucleotide in cells was estimated to be over 2.9 mM (between 2.9 and 5.1 mM) at 95% confidence limits from three different experiments (Figure 6C). We estimated the cytoplasmic concentration of ATP alone using a luciferase assay (see the Methods section) and obtained a value of 2.0 ± 0.2 mM following addition of glucose to the cells. This value is somewhat below the estimate of total adenine nucleotide concentration. These estimates are in excellent agreement with previous esti-

mates based on analysis of cell extracts of *S. cerevisiae*[38] which show a total adenine-nucleotide concentration of 4 mM and an average concentration of ATP of 2.1 mM under the same conditions as those used here. The inability of the ATP aptamer to differentiate between ATP and ADP did not allow us to track temporal dynamics of ATP levels in cells, but we have nevertheless shown that AptaNPs can be used to determine total concentration of adenine nucleotides in the cytoplasm. Moreover, the dynamic range of aptamer switch probe based on DNA aptamer sequence used in this study did not allow an accurate determination of adenine-nucleotide concentration. But, our results showed that aptamer switch probes can be used for *in vivo* determination of dynamics of metabolites in cytoplasm. Considering the success of aptamer-based fluorescent probes for small molecule recognition and the use of nanoparticle-confined fluorescent sensors in literature, aptamers embedded in nanoparticles are proposed as an excellent biorecognition PEBBLE nanosensor for metabolite measurements. AptaNPs can be robust *in vivo* sensors providing the necessary stability and protection for aptamer-target recognition for a real-time monitoring of metabolites in cells, tissues, or organisms. Given a selected aptamer for the desired target, AptaNPs can be prepared in a single-step process. The AptaNPs consist of a nonbiodegradable porous polymer matrix that can transport water-soluble aptamer probes into regions of interest inside cells. The porous nature of the polyacrylamide particles allows rapid diffusion of the target of interest to interact with probes inside the nanoparticle. The small size of the particles enables the target to diffuse rapidly between the matrix and the external solution allowing us to assume equilibrium between the target bound to the aptamer and the target in the exterior solution. Finally, the confinement of aptamer probes prevents interaction between aptamer molecules and nucleases present in the cytoplasm.

CONCLUSION

We have embedded a fluorescent aptamer switch probe responding to adenine nucleotides in 30 nm polyacrylamide nanoparticles. It was demonstrated that the aptamer switch probe in free solution or embedded in nanoparticles responds almost equally to adenine nucleotides. AptaNPs could be transferred into live yeast cells to measure the adenine nucleotide content of cytoplasm. The polyacrylamide nanoparticles were shown to protect the aptamer switch probe from nucleases and allow rapid measurements of external target concentrations. The design as described here can be extended to any small molecule of interest inside live cells by selecting and embedding a specific aptamer in an appropriate nanocapsule. An alternative design for intracellular measurements of metabolites was presented by Zheng *et al.*[39] These promising properties make aptamers embedded in nanoparticles excellent nanosensors for *in vivo* real-time measurements of metabolites. Our future efforts will focus on selecting a new DNA aptamer responding selectively to ATP and using AptaNPs to measure cytoplasmic ATP concentration dynamics in real-time.

METHODS

Reagents: All reagents were purchased from Sigma (Munich, Germany) except for RNase OUT inhibitor which was obtained from Invitrogen (Paisley, UK). All oligonucleotides were synthesized by VBC-Biotech (Vienna, Austria).

Nanoparticle Synthesis and Characterization. Polyacrylamide NPs were prepared by an inverse microemulsion polymerization reaction modified from Daubresse.[40] Briefly, 3.08 g of dioctyl sulfosuccinate (AOT) and 1.08 g of Brij 30 was dissolved in 43 mL of hexane and deoxygenated by sonication for 1 h. Meanwhile a 38.8%T solution of acrylamide (1.35 g of acrylamide and 0.4 g of *N,N*-methylenebisacrylamide was dissolved in 4.5 mL of 10 mM sodium phosphate buffer pH 7.25), 30 nmol of ATP-ASP or control-ASP and 15 μL of Oregon Green dextran (10 mg/mL) was added. A 2.0 mL portion of this solution was added dropwise to the hexane solution and left for 20 min under argon to form the microemulsion. Polymerization was initiated by the addition of 50 μL of a 10% w/v solution of sodium bisulfite, and the reaction was allowed to proceed for 3 h. The hexane was removed in vacuum, and the nanosensors were precipitated by the addition of 100 mL of ethanol. The suspension was transferred to an Amicon ultrafiltration cell model 2800 (Millipore Corp., Bedford, USA), filtered through a 100 kD filter, and washed with 4 × 100 mL of ethanol to remove unreacted monomers and surfactants. The particles were resuspended in 50 mL of ethanol, filtered (0.025 μm nitrocellulose filter membrane), and dried in vacuum; 380 mg of dry nanoparticles was obtained. Vacuum dried particles were kept at −20 °C until they were used in experiments. The nanoparticles were used in all experiments within 1 h after resoloubilization. NP size was obtained by a dynamic light scattering method as 30 nm (15 mW laser, incident beam 633 nm; Brookhaven Instruments Corporation, Holtsville, NY) in a non-negative least-squares curve fit analysis. The measurements were performed in triplicate at room temperature. The average number of aptamer sensor in each nanoparticle was estimated as 0.4.

Aptamer and Nanosensor Response Analysis. Free solution ASPs (50 nM) or AptaNPs (0.5 mg/mL) were prepared in cytosol buffer (see below). The nanoparticles were solubilized by sonication. Fluorescence spectra were recorded at 25 °C unless otherwise indicated. ATP or other target molecules were added directly to the assay solution which was continuously stirred using a magnetic stirrer. A representative cytosol buffer (20 mM phosphate buffer, pH = 7.3, 140 mM KCl, 10 mM NaCl, 2% BSA, 1 mM $MgCl_2$, 5.5% glucose), based on Godt *et al.*[41] to mimic cell cytoplasm, was used.

Nuclease Degradation and Leaching Assays. Target titration experiments were performed in the presence or absence of RNase-OUT inhibitor to evaluate the effect of degradation on RNA aptamer switch probes. For DNase degradation, 1085 units of DNase I was added to a 100 nM solution of free aptamer in assay buffer (2 mL) and a 1 mg/mL solution of AptaNPs in assay buffer (2 mL), and the fluorescence was monitored continuously for 90 min to follow the effect of degradation. Leaching experiments were performed using dialysis cassettes of MWC = 20000 (Thermo Fischer Scientic Inc., Rockford, USA). A 1.0 mL portion of a 50 mg/mL solution of AptaNPs was placed in the cassette according to manufacturer's procedures and left in 200 mL of as-

say buffer. The fluorescence of the surrounding buffer was followed for 72 h along with a solution of aptamer probe of equal fluorescence as a control.

Delivery of NPs into Yeast Cells. Yeast cells, *Saccharomyces cerevisiae* diploid strain X2180, were grown and harvested as described in Poulsen *et al.*[42] The cells were starved for 3 h at room temperature in 100 mM sodium phosphate buffer, pH 6.8, before use. Nanoparticles, pretreated with DNase I, were inserted into yeast cytoplasm by electroporation.[33] The electroporated cells were washed three times and used immediately in fluorimeter and microscopy measurements. It is previously observed that X2180 cells maintained 95% viability after insertion of polyacrylamide nanoparticles at similar size.[33]

Measurements of Intracellular ATP. Measurements of the average intracellular ATP concentration were made by first quenching the cells with boiling buffered ethanol and subsequently extracting the metabolites as described by Gonzales *et al.*[43] The ATP concentration in the extract was then measured using the Biaffin (Kassel, Germany) ATP determination kit (luciferase). The protein content of the cells was determined according to Lowry *et al.*[44] The average intracellular concentration of ATP was determined assuming an intracellular volume of 3.75 μL per mg of protein.[38]

Spectrofluorometry, Confocal Fluorescence Microscopy, and Flow Cytometry. To visualize cellular uptake and distribution of the aptamer probe containing nanoparticles, fluorescence emission spectrum of Texas Red (excitation/emission 580 nm/600 nm) was detected in the red channel (545 nm/590LP nm) of a Zeiss LSM 510 META (Jena, Germany) confocal microscope with an oil immersion objective (×63, NA 0,7) on live cells. The fluorescence of aptamer probes was measured in a FS920 steady-state spectrofluorimeter from Edinburgh Instruments (Edinburgh, Scotland) equipped with a 450-W xenon arc lamp. Texas Red was excited at 580/3 nm and emission was measured at 600/6 nm. Oregon Green was excited at 480/1 nm and emission was measured at 520/2 nm. For flow cytometry assays, at least 3000 events were counted using a cell chip in a Bioanalyzer 2100 (Agilent, Santa Clara, USA). The results of flow cytometry were analyzed in FlowJo (Tree Star, Inc., Ashland, OR).

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Supporting Information Available: Figures for size determination by DLS, RNA−ATP-ASP binding analysis, studies of leaching of the aptamer from nanoparticles, melting temperature analysis of aptamer switch probes, and flow cytometry analysis. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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