

## Chemicals, Experimental Section and Figures

### Chemicals

All used solvents were of analytical-reagent grade and no further purifications were performed. Ethanol, methanol and dichloromethane were purchased from Roth Chemicals (Karlsruhe, Germany) while 2-methoxyethanol and anhydrous dimethylformamide were purchased from Aldrich (St. Louis, MO, USA).

For the synthesis of **4.Zn**, 4-bromo-1,8-naphthalic anhydride, 6-aminohexanoic acid, di-*tert*-butyl dicarbonate (di-Boc), 2-chloromethylpyridine and potassium iodide were all purchased from Aldrich.

For the silylation of the sulforhodamine derivative and synthesis/functionalization of silica nanoparticles, tetraethylorthosilicate (TEOS), aminopropyltriethoxysilane (APTEOS), ammonium hydroxide (NH<sub>4</sub>OH, 25% w/w in water) *N,N*-diisopropylethylamine and sulforhodamine B acid chloride were supplied by Fluka and 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) was acquired from Iris Biotech.

For the fluorescence measurements, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adenosine 5'-triphosphate disodium salt hydrate (ATP), guanosine 5'-triphosphate sodium salt hydrate (GTP), cytidine 5'-triphosphate disodium salt (CTP) and uridine 5'-triphosphate trisodium salt hydrate (UTP) were purchased from Aldrich.

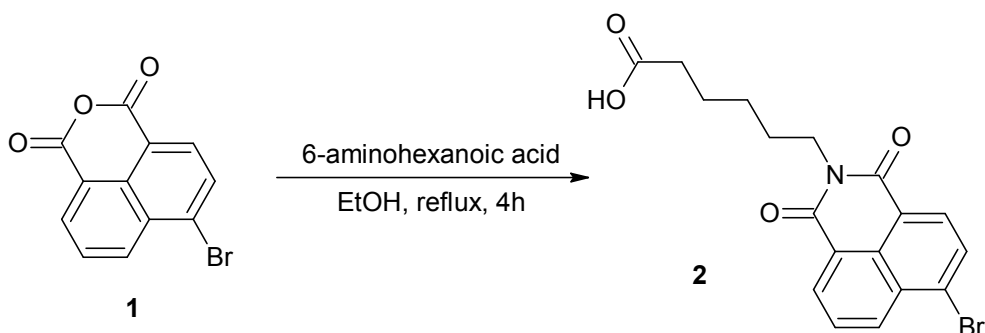
For experiments with living cells we used the adherently growing cell line NRK-52E purchased from the German Resource Centre for Biological Materials (DSMZ, Braunschweig, Germany). Cells were grown in Dulbecco's modified Eagle's medium with 4.5 g/L D-glucose supplemented with 5 % (v/v) fetal calf serum, 100 µg/mL penicillin/streptomycin and 2 mM L-glutamine, all purchased from Biochrom (Berlin, Germany). All routine subculturing was performed by standard trypsinization protocols using 0.25% (w/v) trypsin plus 1 mM EDTA (Biochrom, Berlin, Germany).

### Experimental Section

#### Synthesis of 4

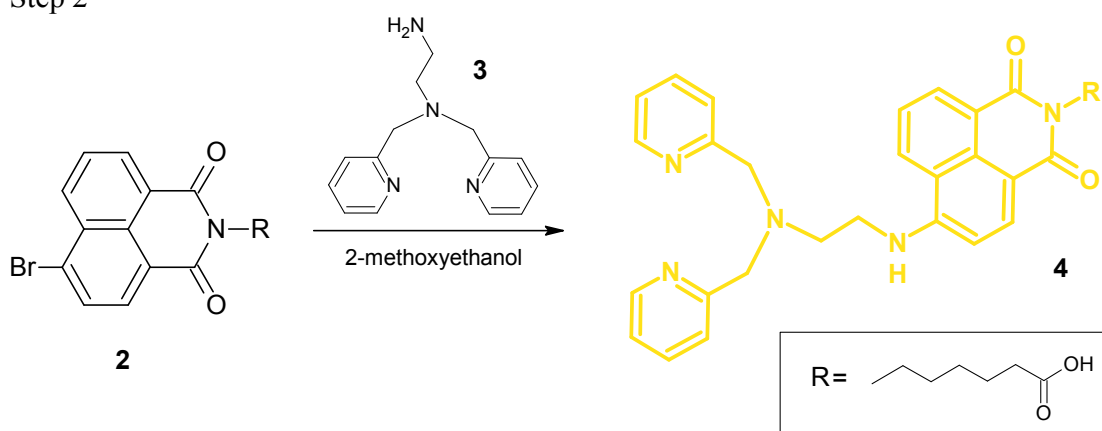
The synthesis was performed in two steps as reported previously.<sup>1</sup> In detail:

Step 1



In a 250 mL round bottom flask, 3 g of 4-bromo-1,8-naphthalic anhydride **1** (10.8 mmol) and 1.43 g of 6-aminohexanoic acid (10.9 mmol) were refluxed in 100 mL of ethanol. The reaction is complete when the initially milky suspension becomes a brown clear solution. Upon cooling, crystals of the product are formed. The gathered crystals were purified through recrystallisation in ethanol. In the end, 2.12 g of **2** were obtained (Yield=50%).  $^1\text{H}$  NMR (250 MHz, MeOD)  $\delta$  (ppm): 8.34 (d, 1 H, H-Aryl-); 8.10 (d, 1 H, H-Aryl-); 7.89 - 7.57 (m, 3 H, H-Aryl-); 4.10 (t, 2 H, -N-CH<sub>2</sub>-); 2.32 (t, 2H, -CH<sub>2</sub>-C=O); 1.71 (m, 4 H, -CH<sub>2</sub>-); 1.45 (m, 2 H, -CH<sub>2</sub>-).  $^{13}\text{C}$ -NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  (ppm): 178.5, 163.60, 163.58, 133.23, 132.04, 131.23, 131.09, 130.65, 130.22, 129.01, 128.06, 123.11, 122.25, 40.24, 33.68, 27.64, 26.48, 24.33.

## Step 2



In a 50 mL round bottom flask, 0.11 g of **2** (0.28 mmol), 0.25 g of *N*-bis-pyridin-2-ylmethylethane-1,2-diamine **3** (1 mmol) and 1 mL of triethylamine were refluxed in 15 mL of methoxyethanol. The reaction was monitored through TLC (Eluent: dichloromethane/ methanol 5:1). When the reaction was completed, the solvent was removed by heating. The crude product was then purified through column chromatography, with increasing solvent polarity using a dichloromethane/methanol mixture (from 5:1 until 3:1). In the end, 80 mg (Yield=52%) of pure **4** were obtained.  $^1\text{H}$  NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 8.68 (d, 1H,  $^3J=8.4\text{Hz}$ ); 8.49 (d, 2H,  $^3J=4.3\text{Hz}$ ); 8.42 (d, 1H,  $^3J=7.3\text{Hz}$ ); 8.15 (d, 1H,  $^3J=8.5\text{Hz}$ ); 7.85 (m, 1H); 7.70-7.63 (m, 2H); 7.50 (d, 2H,  $^3J=7.8\text{Hz}$ ); 7.22 (m, 2H); 6.64 (d, 1H,  $^3J=8.6\text{Hz}$ ); 3.98 (t, 2H); 3.89 (s, 4H); 3.51 (m, 1H, Ar-NH); 2.85 (t, 2H); 2.12 (t, 2H); 1.56 (m, 4H); 1.32-1.23 (m, 4H).  $^{13}\text{C}$  NMR (100

MHz, DMSO- $d_6$ )  $\delta$  (ppm): 164.2, 163.3, 159.6, 150.9, 149.3, 136.9, 134.5, 131.1, 129.9, 128.7, 124.8, 123.3, 122.6, 122.4, 122.3, 120.6, 108.1, 104.3, 60.1, 51.5, 41.1, 35.4, 28.0, 26.8, 25.3. MS (Micro-ESI): 550.5 (M-H).

#### Preparation of surface amino-functionalized rhodamine-doped silica nanoparticles

In order to achieve covalent attachment of the reference dye to the silica matrix of the nanoparticles, a silylated-sulforhodamine derivative was previously prepared by conjugation of the silica precursor APTEOS with the sulfonyl chloride function of sulforhodamine B acid chloride according to a described procedure.<sup>3</sup> This sulforhodamine derivative was then used for the synthesis of the fluorescent silica nanoparticles. The full procedure was performed through a modified Stöber method with subsequent surface functionalization with amine groups.<sup>4</sup> In detail:

##### *Synthesis of the rhodamine-doped silica nanoparticles (cores)*

To a polypropylene flask containing 150 mL ethanol, the silylated sulforhodamine derivative (40 mL of the above solution), the ammonium hydroxide aqueous solution (7.5 mL) and TEOS (5 mL) were sequentially added. The mixture was stirred vigorously at room temperature overnight. The resulting colloidal suspension was then thoroughly washed via repeated cycles of centrifugation and re-dispersion in EtOH. An undoped silica shell was then coated through a seed-growth technique, by addition of TEOS (0.5 mL) and an ammonium hydroxide aqueous solution (0.5 mL) to an ethanol suspension of 100 mg of the initial rhodamine-doped nanocores at 10 mg/mL. The suspension was allowed to stir overnight at room temperature. The resulting colloidal suspension was thoroughly washed via repeated cycles of centrifugation and re-dispersion in EtOH. The resulting ethanol suspension of nanoparticles was filtered through a 1  $\mu\text{m}$  pore diameter PTFE filter.

##### *Surface amino-functionalization of the rhodamine-doped silica cores*

To an ethanol suspension of the previously prepared rhodamine-doped silica cores (5 mg/mL) APTEOS (1 mmol/g) was added. After subsequent addition of pyridine in a catalytic amount, the suspension was allowed to stir under reflux overnight. The resulting amino-functionalized fluorescent nanoparticles were washed three times via cycles of centrifugation and ultrasonic re-dispersion in EtOH.

##### *Immobilization of 4.Zn onto the surface amino-functionalized silica cores (finalized nanoparticles/nanoprobes)*

The ATP-chemosensor complex **4.Zn** was generated *in-situ* during the NHS-ester activation of the carboxylic acid. In detail: in a plastic vial, 2.2 mg of **4**, 9.0 mg of zinc perchlorate and 4.2 mg of TSTU were dissolved in 5 mL of DMF. The mixture was then submitted to a sonication bath for 1 minute and left another 15 minutes for stirring. To the reaction mixture, 2.5 mL of an ethanol solution of amine surface-functionalized silica nanoparticles (20 mg/mL) were added (this solution was sonicated just before addition, to ensure the homogeneity upon volume transferring) and the reaction mixture was left to

stir for 48 hours at room temperature. Afterwards, the vial was centrifuged (20 minutes at 9000 r.p.m.), the supernatant was removed and the solids were resuspended in 10 mL of methanol. Cycles of centrifugation/resuspension were repeated 4 times with methanol and 4 times with ethanol. The supernatants were monitored under UV light until there was no visible fluorescence left (which happened after the third methanol washing step). Then, the solvent was changed gradually to water by removing half of the supernatant and replenishing the volume with water up to 10 mL. This procedure for solvent exchange was performed in three turns, with cycles of centrifugation/resuspension in between. Finally, the resulting nanoparticles were stored suspended in water. Prior to every fluorescent measurement, the nanoparticles were resuspended 5 minutes in sonication bath.

### Characterization of ATP-sensing nanoparticles

All aqueous solutions and buffers were prepared using deionized double distilled water (conductivity  $\leq 0.055 \mu\text{S cm}^{-1}$ ) produced by Seralpur PRO 90 CN system (Seral, Ransbach-Baumbach, Germany). The fluorescence spectra for all samples were obtained on a Fluorolog 3 from Jobin Yvon-Spex at a temperature of  $(25 \pm 1)^\circ\text{C}$ . Steady-state fluorescence measurements of free dye **4.Zn** were performed as follows: an aliquot (50  $\mu\text{L}$ ) of a solution of **4.Zn** in methanol (0.3 mM) was dissolved in 2950  $\mu\text{L}$  of HEPES buffer 0.1 M at pH 7.4 in a quartz cuvette (10 mm). Afterwards, small aliquots of the anionic analyte (up to a total volume of 100  $\mu\text{L}$ ) were added to the cuvette and fluorescence spectra were acquired between each addition. The value and polydispersity of the hydrodynamic diameter of the silica nanoparticles were determined in an ethanolic suspension through dynamic light scattering (second order cumulant analysis) using an ALV-NIBS/HPPS system with an external ALV-5000/EPP correlator (ALV-GmbH, Langen, Germany). Steady-state fluorescent measurements of functionalized nanosensors were performed by adding 100  $\mu\text{L}$  of an aqueous suspension of nanoparticles with a concentration of  $1 (\pm 0.2) \text{ mg/mL}$  to a quartz cuvette containing 2900  $\mu\text{L}$  of HEPES 0.1 M with pH previously adjusted to 7.4. The emission spectra for both indicator dye ( $\lambda_{\text{Exc}} = 450 \text{ nm}$ ) and Rhodamine ( $\lambda_{\text{Exc}} = 530 \text{ nm}$ ) were acquired separately. Afterwards, small aliquots of the anionic analyte (up to a total volume of 100  $\mu\text{L}$ ) were added to the cuvette and fluorescence spectra were acquired between each addition. For the studies with ethylenediamine tetraacetate sodium salt (purchased from Aldrich), an aqueous solution of 0.1 M EDTA was used. The excitation wavelengths for all **4.Zn-NPs** samples were 450 nm (for **4.Zn**) and 530nm (for rhodamine), with a slit width set to 2 nm bandpass.

### Confocal Laser Scanning Microscopy

Normal rat kidney (NRK) cells were grown to confluence in an 8-well chamber that was prepared by gluing an 8-well chamber top to the bottom of a regular Petri dish by means of a silicon adhesive. Before microscopic inspection the cells were incubated with the ATP-sensing nanoparticles at 0.2 mg/mL for 48 hours in serum-containing medium at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  atmosphere. At the end of the incubation period, the cells were thoroughly washed with phosphate buffered saline supplemented with 0.5 mM  $\text{Mg}^{2+}$  and

1 mM  $\text{Ca}^{2+}$  ( $\text{PBS}^{++}$ ). Afterwards the 8-well chamber top was removed, the dish was flooded with  $\text{PBS}^{++}$  and the cells were studied with the upright fluorescence microscope 90i (Nikon) additionally equipped with the confocal laser scanning unit C1 (Nikon). For nanoparticle imaging we used laser sources with emission wavelengths of 408 nm (4.Zn) and 543 nm (reference dye), respectively. Emission of 4.Zn was collected through a 515/30 nm bandpass filter, whereas a 650 nm longpass filter was used for the reference dye. After confocal microscopic inspection of the living cells, they were fixed with 4% paraformaldehyde in  $\text{PBS}^{++}$  for 10 min, stained with 10 ng/mL DAPI in  $\text{PBS}^{++}$  for 10 minutes at RT, washed and inspected again. DAPI fluorescence was excited at 408 nm and recorded through a 450/35 nm bandpass filter. All microscopic imaging was pursued using a 60x water dipping objective (NA=1.0).

### ECIS

Impedimetric monitoring of NRK cells during the exposure to ATP-sensing nanoparticles is based on the well established ECIS™ technology.<sup>5</sup> The measurement chambers consist of a commercial 8-well cell culture dish with gold electrodes deposited on the bottom of each well (Applied BioPhysics Inc. (USA); type 8W1E). Each well of these electrode arrays contains a small working electrode (area:  $5 \times 10^{-4} \text{ cm}^2$ ) and a large counter electrode (area:  $\approx 0.15 \text{ cm}^2$ ). The 8-well electrode array is placed in a humidified cell culture incubator at 37 °C with 5 %  $\text{CO}_2$ . Electrodes are interfaced to the electronic equipment (ECIS 1600R) located outside the incubator by a holder manufactured by Applied BioPhysics Inc (Troy, NY). Relays allow switching between the different electrodes. Relays and impedance analyzer are connected to an ordinary PC that controls the experiment. During exposure to the ATP-sensing nanoparticles the cells have been monitored in MFT mode, i.e. the impedance and phase are sampled continuously at 11 discrete frequencies between 25 Hz and 60 kHz using entirely non-invasive AC currents of less than 1  $\mu\text{A}$ . The time resolution of the measurement was better than 5 minutes for 16 different samples followed simultaneously. The impedance magnitude at a sampling frequency of 32 kHz was used as cytotoxicity indicator as it sensitively detects any contraction and detachment of the intact cell body from the electrode surface.<sup>6</sup> The viability index after an exposure time of 48 hours was calculated from the recorded impedance data by the following relationship:

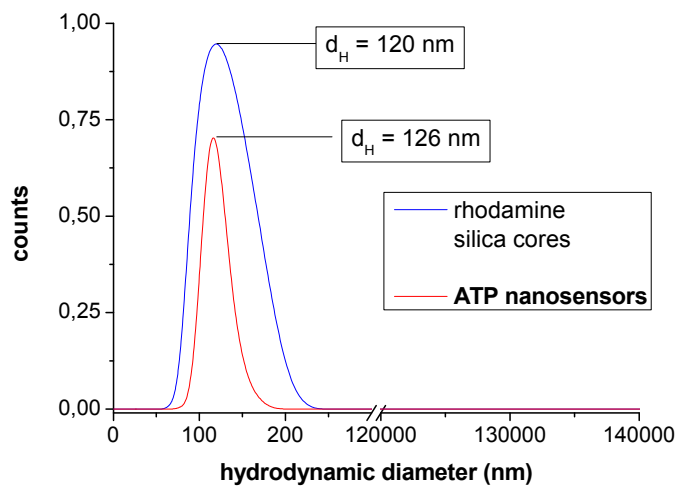
$$\text{Viability Index [\%]} = (|Z|_{\text{exp}} - |Z|_{\text{ce}}) / (|Z|_{\text{nc}} - |Z|_{\text{ce}})$$

with  $|Z|_{\text{exp}}$ : Impedance Magnitude of sample under study  
 $|Z|_{\text{ce}}$ : Impedance magnitude of cell-free electrode  
 $|Z|_{\text{nc}}$ : Impedance magnitude of negative control (untreated cells)

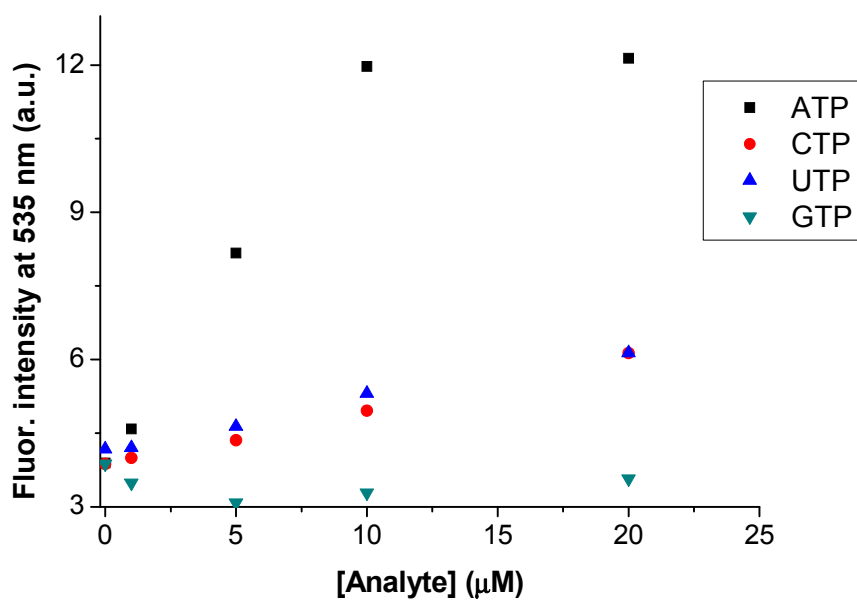
### References

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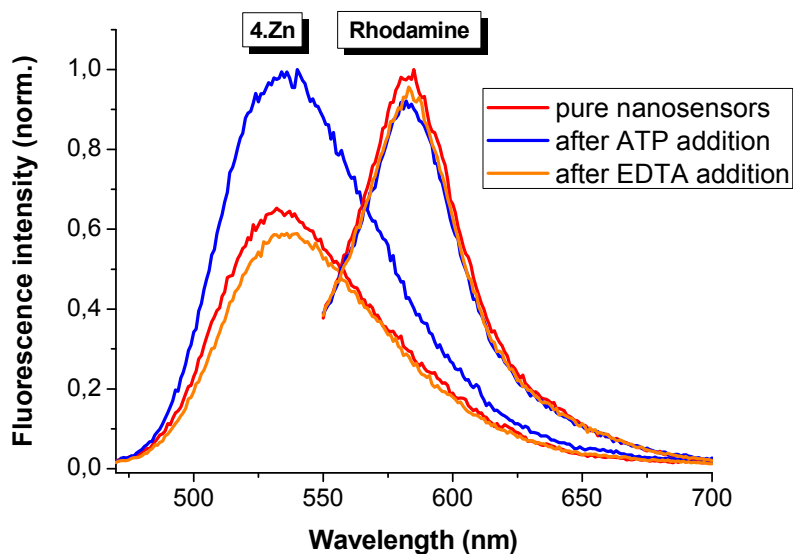
## Figures



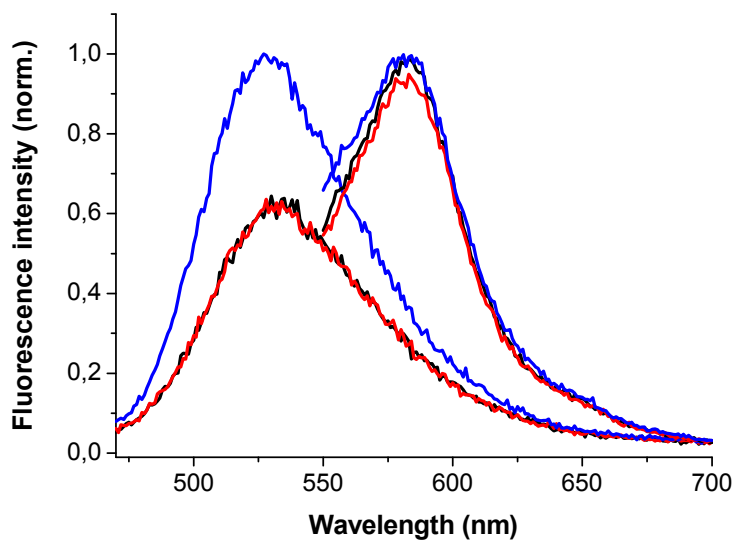
**Fig. S1.** DLS measurements of rhodamine-doped silica cores (blue line) and the surface-functionalized ATP nanosensors (red line) in ethanol. PDI values were 0.04 and 0.07 ( $\pm 0.01$ ), respectively.



**Fig. S2.** Changes in fluorescence intensity of **4.Zn** ( $5\mu\text{M}$ ) upon exposure to different concentrations of triphosphorylated nucleotides ( $\lambda_{\text{exc}}=450\text{nm}$ ,  $\lambda_{\text{em}} = 535$  nm).

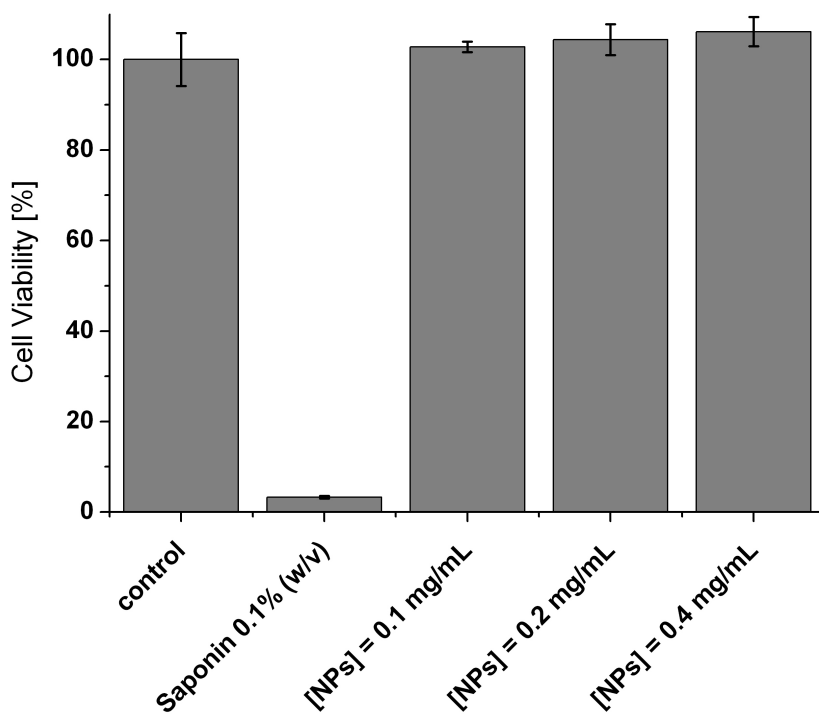


**Fig. S3.** Changes in fluorescence of the nanosensors upon successive addition of ATP and EDTA ( $\lambda_{\text{exc}}$  (4.Zn) = 450 nm,  $\lambda_{\text{exc}}$  (Rhod.) = 530 nm). Description: **red lines** – pure nanosensors; **blue lines** – nanosensors with 333  $\mu\text{M}$  ATP; **orange lines** – nanosensors with 1667  $\mu\text{M}$  EDTA (after addition of ATP).



**Fig. S4.** Changes in fluorescence of the nanosensors upon successive addition of ATP and Zn (II) in HEPES buffer 0.1M at pH 7.4 ( $\lambda_{\text{exc}}$  (4) = 450 nm,  $\lambda_{\text{exc}}$  (Rhod.) = 530 nm). Description: **black lines** – pure nanosensors; **red line** – nanosensors with 333  $\mu\text{M}$  ATP; **blue line** – nanosensors with 3330  $\mu\text{M}$  Zn (II) (after ATP addition).





**Fig. S5.** Cell viability data of the ATP-sensing nanoparticles at different concentrations after 48 hours obtained from cultured NRK cells using impedance-based measurements (32 kHz) relative to untreated cells (set to 100%). As positive control cells were exposed to 0.1% (w/v) Saponin.