Supporting information

**Triggered Release of Loads from Microcapsule-in-Microcapsule Hydrogel Microcarriers: En-Route to an “Artificial Pancreas”**

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Experimental Section

Reagents and materials

Magnesium chloride, sodium chloride, 4-carboxyphenylboronic acid, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES base), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES acid), sodium phosphate dibasic, sodium phosphate monobasic, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), ammonium persulfate (APS), N,N,N’,N’-tetramethylethylenediamine (TEMED), acrylamide solution (40%), polyallylamine hydrochloride (PAH, MW = 58 kDa), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), glucose oxidase, insulin, catalase, zinc chloride, carboxymethyl cellulose (CMC, medium viscosity, D.S 0.9), rhodamine B isothiocyanate, fluorescein isothiocyanate isomer I (FITC), 7-hydroxycoumarin-3-carboxylic acid Nsuccinimidyl ester were purchased from Sigma-Aldrich. Tetramethylrhodamine-dextran (TMR-D, MW = 70 kDa) was purchased from Life Technologies Corporation (USA). CdSe/ ZnS (2.25nm) QDs purchased from Evident technologies. All oligonucleotides were synthesized, purified by standard desalting, and freeze-dried by Integrated DNA Technologies, Inc. (Table S1, ESI). Ultrapure water from a NANOpure Diamond (Barnstead International, Dubuque, IA) source was used in all experiments. A Magellan XHR 400L scanning electron microscope (SEM), FV-1000 confocal microscope (Olympus, Japan) and Focused Ion Beam (FIB) Helios NanoLab™ 460F1 DualBeam™ were employed to characterize the microparticles. Glucometer Accutrend plus kit mg/dl with Accutrend Glucose II 25 STR strips (Roche).

Synthesis of acrydite-modified oligo/acrylamide copolymers

100 μL of a solution consisting of 0.75 mM acrydite-modified oligonucleotides ([2] and H1 or (x) in a ratio of 2:1) and 1.5 % acrylamide was bubbled with nitrogen for 3 min, followed by the addition of 7.5 μL of initiator mixture (prepared by 10 mg APS in 5 μL TEMED and 95 μL H2O). The resulting solution was subjected to additional 5 min of nitrogen bubbling, followed by incubation at 4 °C for 12 h to form the copolymer chains P1 and P2. Polymer P1 ([2] and H1) was purified and separated from the unreacted compounds using a 30k MWCO Amicon filter, whereas for polymer P2 (2) and (x)) a 10k MWCO Amicon filter was used. After being washed with water three times, the copolymer solutions were dried under a gentle flow of nitrogen gas and re-dispersed in buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl2). To polymer P2, after the determination of the concentration of (x), hairpin H2 was added in a molar ratio of 1:1. The polymer solutions were incubated at 95 °C for 5 min, followed by incubation on ice for 30 min to ensure the efficient closing of the hairpins.

Synthesis of 5'-Amino Modifier C6-modified oligo/Carboxymethyl cellulose (CMC) copolymers

2 mL of a MES buffer solution (10 mM, pH 5.5), containing CMC, 20 mg, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride, 20 mg, were incubated for 5 minutes and then sulfo-N-hydroxysuccinimide, 26 mg, was added and the solution was incubated for additional 10 minutes. To the resulting solution, 2 mL of HEPES buffer (50 mM, pH 7.2) containing the amine-functionalized nucleic acids (300 μM of H1 or (x) and 600 μM of (2)), were added. The mixture was gentle shacked for 2
h at room temperature. The modified polymers, $P_1$ ($\mathbf{2}$ and $H_1$) and $P_2$ ($\mathbf{2}$ and $x$), were purified and separated from the unreacted compounds using MWCO 10K Amicon spin filters. After being washed with water three times, the copolymer solutions were dried and re-dispersed in buffer (10 mM HEPES, pH 7.0, containing 25 mM MgCl$_2$). To polymer $P_2$, after the determination of the concentration of $x$, hairpin $H_2$ was added in a molar ratio of 1:1. The polymer solutions were incubated at 95 °C for 5 min, followed immediately by incubation on ice for 30 min to ensure the efficient closing of the hairpins.

The MPA-capped CdSe/ZnS QDs were prepared as it was described in our previous work.$^1$

The insulin/GOx/catalase-fluorophore conjugates were prepared as it was described previously by G.Sitta Sittampalam et.al.$^2$ GOx was modified with fluorescein isothiocyanate isomer I (FITC) ($\lambda_{\text{ex}}$=490nm; $\lambda_{\text{em}}$ = 525nm) or with 7-hydroxycoumarin-3-carboxylic acid N-succinimidyl ester (Coumarin) ($\lambda_{\text{ex}}$=360nm; $\lambda_{\text{em}}$ = 410nm), catalase was modified with rhodamine B isothiocyanate (Rhodamine) ($\lambda_{\text{ex}}$=544nm; $\lambda_{\text{em}}$ = 576nm) and insulin was modified with 7-hydroxycoumarin-3-carboxylic acid N-succinimidyl ester (Coumarin) ($\lambda_{\text{ex}}$ =360nm; $\lambda_{\text{em}}$ = 410nm) or with fluorescein isothiocyanate isomer I (FITC) ($\lambda_{\text{ex}}$=490nm; $\lambda_{\text{em}}$ = 525nm).

Preparation of CaCO$_3$ microparticles with different loads

CaCO$_3$ particles were prepared by a precipitation reaction between equal amounts of CaCl$_2$ and Na$_2$CO$_3$ under magnetic stirring at room temperature. CaCO$_3$ particles loaded with TMR-D were obtained through coprecipitation by mixing CaCl$_2$ (307 μL, 0.33 M) and Na$_2$CO$_3$ (307 μL, 0.33 M) solutions, in the presence of TMR-D (30 μL, 6.25 mg mL$^{-1}$). The final volume was adjusted to 1020 μL by addition of deionized water. After magnetic stirring for 110s, the suspension was left for 70s at room temperature to settle down. The particles were centrifuged at 100 rcf for 20s, followed by the removal of the supernatant solution, and the subsequent resuspension of the particles in water. This washing procedure was repeated twice in order to remove the byproducts resulting from the precipitation reaction.

GOx or GOx and catalase loaded microparticles were prepared as described above (GOx: 30 μL, 1 mg mL$^{-1}$, catalase: 30 μL, 24 mg mL$^{-1}$)

Synthesis of DNA–acrylamide/CMC hydrogel microcapsule-in-microcapsule

The CaCO$_3$ microparticles were suspended in 600 μL of 1 mg mL$^{-1}$ PAH solution (10 mM HEPES, pH 7.2, containing 25 mM MgCl$_2$) and kept under continuous shaking for 30 min. The PAH-coated particles were washed twice with buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl$_2$), followed by centrifugation at 100 rcf for 20 s. Subsequently, the PAH-coated microparticles were incubated with 600 μL of the promoter nucleic acid (1) (final concentration 10 μM) and kept under continuous shaking at room temperature for 30 min. After being washed twice with buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl$_2$), followed by centrifugation at 100 rcf for 20 s, the DNA hydrogel particles were prepared by mixing the polymer sets ($P_1$ and $P_2$) and the nucleic acid sequences (3) and (4) (or only (3) for the microcapsule-in-microcapsule with GOx or GOx and catalase) with the promoter-coated CaCO$_3$
microparticles. The final concentration of each hairpin was 10 μM. The particles were incubated overnight (approximately 12h) at room temperature under continuous shaking, followed by centrifugation at 100 rcf for 20 s to remove non-adsorbed polymers and the subsequent resuspension in buffer (10 mM HEPES, pH 7.0, containing 25 mM MgCl₂). This washing procedure was repeated twice.

In order to create the CaCO₃ interlayer, the one-layer DNA–acrylamide/CMC hydrogel particles were suspended in buffer solution (37.5 μL, 10 mM HEPES, pH 7.2, containing 25 mM MgCl₂) and 337.5 μL of water, and mixed with 307 μL of CaCl₂ 0.33M and 307 μL of Na₂CO₃ 0.33M, in the presence of CdSe/ZnS QD (30 μL, 240 nM). After magnetic stirring for 110 s, the suspension was left for 70 s at room temperature to settle down. The particles were centrifuged at 100 rcf for 20 s, followed by the removal of the supernatant solution, and the subsequent resuspension of the particle suspension in water. This washing procedure was repeated twice in order to remove byproducts resulting from the precipitation reaction. Then, the outer hydrogel layer was prepared using the procedure described above for the inner hydrogel layer, adding together with the set of polymers (P₁ and P₂) the nucleic acid sequences (5) and (6) for the i-motif system and (7) for the G-quadruplex system.

Similarly, the microcapsule-in-microcapsule system containing - GOx or GOx and catalase in the inner core and fluorophore-modified insulin (30 μL of 16 mM solution) in the outer core was prepared as described above excluding the Zn²⁺-ion-dependent DNAzyme (4) in the inner hydrogel layer and exchanging the dyes for the respective enzymes.

**Dissolution of the Core**

120 μL of a 0.1 M EDTA solution (pH 7.5) were added into 60 μL of microparticle solution containing 1700 microparticles/μL and 60 μL of buffer solution (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂). The resulting solution was incubated for 1 h to dissolve the CaCO₃ cores. When the suspension became clear, the capsules were washed with buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂) using slow centrifugation (50 rcf, 20 min) three times.

**Stimuli-induced unlocking of the hydrogel microcapsules-in-microcapsules and release of the encapsulated loads**

**Zn²⁺-ions and pH-responsive system**

120 μL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂ containing 20 μL of DNA–acrylamide\CMC bilayer hydrogel responsive microcapsules-in-microcapsules (1700 microcapsules-in-microcapsules per μL) were added to a cuvette. After the microcapsules-in-microcapsules precipitated, 9 μL of HCl (1 M) and/or 9 μL of ZnCl₂ (1 M) were added to the solution and the release of the respective loads was measured through fluorescence. Fluorescence (TMR-D (λₑₓ = 546nm; λₑᵐ = 580nm), CdSe/ZnS QDs (2.25 nm) (λₑₓ = 464nm; λₑᵐ = 482 nm)).

For the switchable pH-stimulated release of CdSe/ZnS QDs from the outer layer, 120 μL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂) containing 20 μL of DNA–acrylamide\CMC hydrogel bilayer responsive microcapsules-in-microcapsules (1700
microcapsule-in-microcapsules per µL) were added to a cuvette. After the microcapsule-in-microcapsule precipitated, 9 µL of HCl (1 M) were added to the solution and the release of the QDs was measured through fluorescence. After 10 min the buffer was exchanging by HEPES buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂). This process was repeated 3 times.

For the Zn²⁺ controlled release of TMR-D at pH 5.5 different samples consisting of 120 µL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂), containing 20 µL of DNA–acrylamide\CMC hydrogel bilayer responsive microcapsules-in-microcapsules (1700 microcapsules-in-microcapsules per µL) were added to a cuvette. After the microcapsules-in-microcapsules precipitated, 9 µL of HCl (1 M) were added to each sample and the release of the respective loads was measured through fluorescence (TMR-D (λ<sub>ex</sub>=546nm; λ<sub>em</sub> = 580nm ), CdSe/ZnS QDs (2.25nm) (λ<sub>ex</sub> = 464nm ; λ<sub>em</sub> = 482nm )). For the switchable K⁺-stimulated release of CdSe/ZnS QDs from the outer layer, 120 µL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂) containing 20 µL of DNA–acrylamide\CMC hydrogel bilayer responsive microcapsules-in-microcapsules (1700 microcapsules-in-microcapsules per µL) were added to a cuvette. After the microcapsules-in-microcapsules precipitated, 9 µL of CE (1.5 M) and/or 9 µL of ZnCl₂ (1 M) were added to the solution and the release of TMR-D was measured through fluorescence. After 10 min the buffer was replaced by new buffer containing 9 µL of KCl (1 M). This process was repeated 2 times.

Glucose regulated insulin release

Different samples consisting of 120 µL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂) containing 20 µL of CMC hydrogel bilayer microcapsules-in-microcapsules (1700 microcapsules-in-microcapsules per µL) were added to a cuvette. After the microcapsules-in-microcapsules precipitated different concentration of glucose were added (final concentration; 0 mM, 5 mM, 10 mM, 15 mM) to each sample and the release of insulin was measured through fluorescence.

For the switchable release of insulin from the outer layer, 120 µL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl₂) containing 20 µL of CMC hydrogel bilayer microcapsules-in-microcapsules (1700 microcapsules-in-microcapsules per µL) were added to a cuvette. After the microcapsules-in-microcapsules precipitated, 2.4 µL glucose (10 mM final concentration) were added to the solution and the release of insulin was measured through fluorescence. 50 min after reaching the release saturation value, 2.4 µL additional of glucose were added and the release of insulin was switched on again. This process was repeated 2 times (the same procedure was
done for the switching with 7 mM glucose by the addition of 1.68 µL of 500 mM stock solution. The switching was done for 3 cycles).

For the switchable release of insulin with washing step from the outer layer, 120 µL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl$_2$) containing 20 µL of CMC hydrogel bilayer microcapsules-in-microcapsules (1700 microcapsules-in-microcapsules per µL) were added to a cuvette. After the microcapsules-in-microcapsules precipitated, 1.68 µL glucose (7 mM final concentration) were added to the solution and the release of insulin was measured through fluorescence. 35 minutes since the addition of 7 mM glucose, the upper solution was extract and buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl$_2$) was added. This process was repeated 5 times.

**Glucometer**

Detection of the glucose level in the presence of GOx and catalase/insulin microcapsules-in-microcapsules.

100 µL of microcapsules-in-microcapsule were added to 400 µL of buffer (10 mM HEPES, pH 7.2, containing 25 mM MgCl$_2$). The glucose concentration was measured after time intervals of 0 min, 2 min, 5 min, 10 min, 20 min, 30 min, 60 min and 100 min by a glucometer (Accutrend plus kit mg/dL with Accutrend Glucose II 25 STR strips (Roche)).
**Table S1.** The sequences of nucleic acids used in this system.

| Sequence | 5' → 3' |
|----------|---------|
| (1)      | GTAGAAAGAAGGTGTCACAGTT |
| (2) (Acrylamide) | /5Acryd/TTTCTTCATTGGTTT |
| (2) (CMC) | /5AmMC6/TTTTCTTCATTGGTTT |
| (3)      | AAAACAATGAAGAAAAACGACTAGACGTTGAAGGATACCAGGGAAACAATGAAGAAAA |
| (4)      | CCC TGG TAT CTA GTT GAG CTG TCT AGT CGG |
| (5)      | TTTCCCAATCCCAATCCCAATCCCAAACATGAAGA |
| (6)      | TTTGTGATTGGGATTTGGATGTGAACATGAAGA |
| (7)      | AAAACAATGAAGAATCTTCTGGGCGGGCGGGCGGGCGGGACTTAAACATGAAGAAT |
| H₁ (Acrylamide) | /5Acryd/TTTTTTTTTGGTGTATTAATTTATGCTTTAAACACCTTCTTTCT |
| H₁ (CMC) | /5AmMC6/TTTTTTTTTGGTGTATTAATTTATGCTTTAAACACCTTCTTTCT |
| H₂      | CAATTCTCCCACTAAACTGAAGAAGGTGTTTAAATGTTGGCCTCTCCACATCAGGTCCAA |
| x (Acrylamide) | /5Acryd/TTTGGACCGATGGTAGAGC |
| x (CMC)  | /5AmMC6/TTTGGACCGATGGTAGAGC |
| NA₁      | /56-FAM/TTAGGGTTAGTCG |
| NA₂      | /56-FAM/TTTAGGGTTAGGG |
| NA₃      | /56-FAM/TATATTAGGTTAGG |
| NA₄      | TTTGGGTAGGGCGGGTTGGGTTGATA/36-FAM/ |
Table S2. Bi-compartmentalized GOx and catalase/insulin loaded microcapsules acting as an “artificial pancreas” - operation features.

| System                                      | Size of Carrier | Insulin Loading | Glucose Concentration Releasing Insulin                                                                 | Stability | Remarks                                            |
|---------------------------------------------|-----------------|-----------------|---------------------------------------------------------------------------------------------------------|-----------|----------------------------------------------------|
| Glucose-Sensitive Microcapsules$^{3}$       | 5μm             | not mentioned   | 100mM (equivalent to 2000mg/dL) not relevant to diabetic control, release at relevant glucose concentration is missing | 2 weeks   | No switching reported H$_2$O$_2$ not degraded      |
| Polysaccharide Hydrogel Network Particles$^{4}$ | 100mm$^3$ patch | 7.9 to 11.4 wt% per particle | 100mg/dL no significant release 400mg/dL release proceed (very high glucose concentration) release at relevant glucose concentration 80-300mg/dL is not reported. | 4 weeks   | Carrier is degraded, possible immune response      |
| Bicompartamental Triggered Glucose Oxidase/Insulin (Present Study) | 2.6±0.3μm | 1300mg/dL (391U/mL) | Release of insulin complies with the glucose range diabetics controlling 80-100mg/dL - no release In glucose concentration >100mg/dL insulin is efficiently released | 2-3 months (8-12 weeks) | Switchable ON/OFF as long as loaded |

Table S2 compares two reported “artificial pancreas” systems (that to the best of our knowledge represent relevant configurations to our microcapsules-in-microcapsules insulin carriers). It should be noted that several other reports discussed the insulin release or applied non-hydrogel polymers or particles as carriers, yet the glucose stimulated release of insulin from the carriers is irrelevant for controlling diabetes (abnormal high concentration of glucose).

Realizing that our microcapsule-in-microcapsule system includes 1700 microcapsules in 1 μL, and knowing the insulin loading per microcapsule we estimate that ca. 400 units of insulin are present in one milliliter of an aqueous HEPES buffer mixture, pH 7.2, of the microcapsules. Presuming that treatment of high glucose levels, 130-200 mg/dL, requires 5 units of insulin, the injection of 100 μL of the microcapsules will allow the autonomous control of the glucose levels for eight cycles. Saying that and assuming that high glucose levels raise twice a day, 100 μL of microcapsules could autonomously control the glucose levels for four days. Alternatively, if the injected volume is 200 μL, an autonomous control of the glucose levels for eight days could be
achieved. If the deviation in glucose levels increases to four-times daily, the 200 μL microcapsule mixture could autonomously control the glucose levels for four days.

Needless to say, further optimization of the system could be accomplished by increasing the load of insulin as well as by the modification of the hydrogel coating comprising the capsules.
Figure S1. Determination of the ratio between nucleic acid units and CMC polymer units on the (2)/H₁ (5AmMC6 modified) conjugated copolymer P₁. (A) Absorbance spectra corresponding to different concentrations of CMC in the presence of constant concentration of the amino-modified nucleic acid (2) and H₁ (final concentration 0.5 μM, ratio (2)/H₁ 2:1). Ratio CMC/DNA: (a) 0:1, (b) 10:1, (c) 20:1, (d) 50:1, (e) 100:1, (f) 150:1 (g) 200:1 (h) 250:1 (i) 300:1 (j) 350:1 (k) 400:1 (l) 500:1 (m) 600:1 (n) 700:1. (B) Calibration curve corresponding to the molar ratio of CMC/nucleic acid((2) and H₁) (5AmMC6 modified) vs. the absorbance ratio at 205 nm/260 nm. The absorbance of the modified polymer was measured and the ratio of CMC to nucleic acid strands was calculated to be 60:1.
Figure S2. Determination of the ratio between nucleic acid units and CMC polymer units on the (2)/(x) (5AmMC6 modified) conjugated copolymer P_2 (without H_2). (A) Absorbance spectra corresponding to different concentrations of CMC in the presence of constant concentration of the amino-modified nucleic acid (2) and (x) (final concentration 0.5 µM, ratio (2)/(x) 2:1). Ratio CMC/DNA: (a) 0:1, (b) 10:1, (c) 20:1, (d) 50:1, (e) 100:1, (f) 150:1 (g) 200:1 (h) 250:1 (i) 300:1 (j) 350:1 (k) 400:1 (l) 500:1 (m) 600:1 (n) 700:1. (B) Calibration curve corresponding to the molar ratio of CMC/nucleic acid((2) and (x)) (5AmMC6 modified) vs. the ratio of absorbance at 205 nm/260 nm. The absorbance of the modified polymer was measured and the ratio of CMC to nucleic acid strands was calculated to be 60:1.
Figure S3. (A) $^1$H-NMR spectrum of CMC polymer P$_1$ ((2)/H$_2$). (B) DOSY-NMR spectrum of CMC polymer P$_1$ ((2)/H$_1$).
Figure S4. (A) $^1$H-NMR spectrum of CMC polymer P$_2$ ((2)/(x)). (B) DOSY-NMR spectrum of CMC polymer P$_2$ ((2)/(x)).
Figure S5. Calibration curve corresponding to the diffusion coefficients of a series of CMC polymers of known average molecular weights (Mw= 90 kDa, 250 kDa and 700 kDa). The diffusion coefficients of the respective polymers were derived recording the DOSY spectrum of each of the polymers. (500 KDa for P₁, and 200 KDa for P₂).
Figure S6. (A) Schematic synthesis of polyacrylamide stimuli-responsive nucleic acid-based hydrogel microcapsules-in-microcapsules loaded with two different fluorophores and the unlocking of the system by two different triggers: Zn$^{2+}$-ions and pH, which results in the release of the loads from the different aqueous compartments. (B) Detailed outline of the substrate (3) and Zn$^{2+}$-ions-dependent DNAzyme (4) associated with the inner hydrogel layer. (C) Detailed outline of the outer hydrogel layer comprising the pH-responsive reconfiguration of the (2)/(5)/(6)/(2) supramolecular duplexes into the separated i-motif structure (at pH 5.5, lower stiffness) and the reverse assembly of the (5)/(6) duplex-bridged hydrogel (at pH 7.2, higher stiffness).
Figure S7. Determination of the ratio between nucleic acid units and acrylamide units on the \(2/H_1(5\text{Acryd modified})\) conjugated copolymer \(P_1\). (A) Absorbance spectra corresponding to different concentrations of acrylamide in the presence of constant concentration of the acrydite-modified nucleic acid \(2\) and \(H_1\) (final concentration 0.5 \(\mu\)M, ratio \((1)/H_1 2:1\)). Ratio acrylamide units/DNA: (a) 1:1, (b) 5:1, (c) 10:1, (d) 25:1, (e) 50:1, (f) 75:1 (g) 100:1 (h) 125:1 (i) 150:1 (j) 175:1 (k) 200:1 (l) 250:1. (B) Calibration curve corresponding to the molar ratio of acrylamide/ nucleic acid \((2)\) and \(H_1\) \(5\text{Acryd modified})\ vs. the ratio of absorbance at 205 nm/260 nm. The absorbance of the modified polymer was measured and the ratio of acrylamide units to nucleic acid strands was calculated to be 30:1.
**Figure S8.** Determination of the ratio between nucleic acid units and acrylamide units on the (2)/(x) (5Acryd modified) conjugated copolymer P₂ (without H₂). (A) Absorbance spectra corresponding to a different concentration of acrylamide in the presence of constant concentration of the acrydite-modified nucleic acid (2) and (x) (final concentration 0.5 µM, ratio (2)/(x) 2:1). Ratio acrylamide units /DNA: (a) 1:1, (b) 5:1, (c) 10:1, (d) 25:1, (e) 50:1, (f) 75:1 (g) 100:1 (h) 125:1 (i) 150:1 (j) 175:1 (k) 200:1 (l) 250:1. (B) Calibration curve corresponding to the molar ratio of acrylamide/ nucleic acid ((2) and (x)) (5Acryd modified) vs. the ratio of absorbance at 205 nm/260 nm. The absorbance of the modified polymer was measured and the ratio of acrylamide units to nucleic acid strands was calculated to be 30:1.
Figure S9. (A) $^1$H-NMR spectrum of polyacrylamide polymer $P_1 ([2]/H_1)$. (B) DOSY-NMR spectrum of polyacrylamide polymer $P_1 ([2]/H_1)$. 
Figure S10. (A) $^1$H-NMR spectrum of polyacrylamide polymer $P_2 ((2)/(x))$. (B) DOSY-NMR spectrum of polyacrylamide polymer $P_2 ((2)/(x))$. 
Figure S11. Calibration curve corresponding to the diffusion coefficients of a series of polyacrylamide polymers of known average molecular (549.4 kDa for P₁, and 390.63 kDa for P₂). The diffusion coefficients of the respective polymers were derived recording the DOSY spectrum of each of the polymers.
Figure S12. Time-dependent release of the loads from the aqueous compartments of the microcapsule-in-microcapsule system at pH 7.2, in the absence of Zn$^{2+}$-ions. No release of the CdSe/ZnS QDs, curve (a), (pH-responsive hydrogel, outer compartment) or TMR-D, curve (b), (Zn$^{2+}$-ion-dependent DNAzyme hydrogel, inner compartment) was detected to the bulk solution.
Figure S13. Triggered time-dependent release of CdSe/ZnS QDs upon subjecting the polyacrylamide bi-compartmentalized microcapsule-in-microcapsule to pH 5.5.
Figure S14. Time-dependent release of CdSe/ZnS QDs upon subjecting the polyacrylamide bi-compartmentalized microcapsule-in-microcapsule to pH 7.2.
Figure S15. Switchable “ON” and “OFF” release of the CdSe/ZnS QDs from the polyacrylamide microcapsule-in-microcapsule system upon the reversible treatment at pH 5.5 and pH 7.2.
Figure S16. Triggered time-dependent release of TMR-D upon subjecting the polyacrylamide bi-compartmentalized microcapsule-in-microcapsule system to different concentrations of Zn$^{2+}$-ions at pH 5.5: (a) 20 mM, (b) 0 mM.
Figure S17. Triggered time-dependent release of the loads (QDs and TMR-D) upon subjecting the polyacrylamide bi-compartmentalized microcapsule-in-microcapsule system to Zn$^{2+}$-ions, 20 mM, and different pH values. (a) Release of TMR-D at pH 7.0, (b) Release of QDs at pH 7.2, (c) Release of QDs at pH 5.5, (d) Release of TMR-D at pH 5.5.
Figure S18. Triggered time-dependent release of TMR-D from the inner compartment of the microcapsule-in-microcapsule system at pH 5.5 in the presence of different concentrations of Zn$^{2+}$-ions (a) 0 Mm, (b) 10 Mm, (c) 20 mM, (d) 30 Mm.
Figure S19. Confocal fluorescence microscopy images corresponding to the release of CdSe/ZnS QDs and/or TMR-D from the bi-compartmentalized-loaded microcapsule-in-microcapsule triggered by pH and/or Zn\(^{2+}\)-ions.

Entry I-The CaCO\(_3\)-loaded bi-compartment particle prior etching.

Entry II- The bi-compartmentalized microcapsule after etching with EDTA.

Entry III- The bi-compartmentalized microcapsule treated with HCl, pH 5.5.

Entry IV- The bi-compartmentalized microcapsule treated with HCl, pH 5.5 and Zn\(^{2+}\)-ions, 20mM.

Colum (a)- Fluorescence image of the red channel (TMR-D).

Colum (b)- Fluorescence image of the green channel (CdSe/ZnS QDs).

Colum (c)- Fluorescence overlay of the red and green channels.

Colum (d)- Bright-field image of the structures.

Entry I- Reveals in (a) and (b) the distinct red fluorescence and green fluorescence of the TMR-D (red) and CdSe/ZnS QDs (green) loaded in the inner and outer compartments of the microparticles-in-microparticles before etching. In (c) the
overlay of (a) and (b) shows distinct separated compartments of (a) and (b). Entry II-
reveals separated red and green compartments corresponding to the bi-compartment
microcapsule-in-microcapsule system after etching. The overlay (c) shows distinct
separated compartments of the red/green fluorophores. The bright field image (d)
shows the two-compartments microcapsules-in-microcapsules. Entry III- (a) and (b)
show the fluorophore image of HCl (pH 5.5) treated microcapsules-in-microcapsules.
As the release of the CdSe/ZnS QDs from the outer layer increase, no green
fluorescence is observed, and only the inner red fluorescence is detected. In (d) the
bright field image confirms the bilayer structure of the microcapsule. In Entry IV- (a)
and (b) shows the fluorescent image of the red/green channels upon treatment of the
microcapsules with HCl (pH 5.5) and Zn$^{2+}$-ions (20mM). No fluorescence is detected in
the two channels or in the overlay channel (c). Nonetheless, the bright field image
confirms the existence of the intact bilayer microcapsule-in-microcapsule structure.
These results confirm that under these conditions the two loads, TMR-D (inner
compartment) and CdSe/ZnS QDs (outer compartment) were release from the
microcapsules.
Figure S20. (A) Synthesis of stimuli-responsive nucleic acid based hydrogel microcapsules-in-microcapsules loaded with two different fluorophores and the unlocking of the system by two different triggers: $\text{Zn}^{2+}$-ions (inner layer, release of TMR-D), $\text{K}^+$-ions/CE (outer layer, release of CdSe/ZnS QDs). (B) Detailed outline of the nucleic acid bridging elements associated with the inner hydrogel layer, $\text{Zn}^{2+}$-ions dependent DNAzyme (3)/(4). (C) Detailed outline associated with the outer hydrogel compartment. Reconfiguration of $\text{K}^+$-ions-stabilized G-quadruplexes (7) into single strands in the presence of CE and the reverse assembly of (7) into the G-quadruplex-bridged units, high stiffness hydrogel layer, in the presence of $\text{K}^+$-ions.
Figure S21. Triggered time-dependent release of the loads from the aqueous compartments of the microcapsule-in-microcapsule system loaded with TMR-D in the inner compartment and CdSe/ZnS QDs in the outer compartment. The compartments are separated by the Zn$^{2+}$-ions-dependent DNAzyme hydrogel (inner compartment). The outer compartment and the bulk solution are separated by K$^+$-ions stabilized G-quadruplex hydrogel. (A) Curve (a) time-dependent release of CdSe/ZnS QDs from the outer compartment in the presence of 8-crown-6-ether (CE), that separate the G-quadruplex crosslinking bridges. Curve (b) shows that the confined TMR-D in the inner compartment was not released. Inset - switchable “ON” and “OFF” release of CdSe/ZnS QDS from the outer compartment by the cyclic treatment of the microparticles with 18-crown-6-ether and K$^+$-ions. (B) Time-dependent release of the loads from the aqueous compartments in the presence of CE and Zn$^{2+}$-ions. Release of CdSe/ZnS QDs, curve (a) and TMR-D, curve (b).
Figure S22. Stepwise release of insulin upon subjecting the bilayer GOx/insulin loaded microcapsules-in-microcapsules to cyclic addition of glucose, 7mM.
Figure S23. Loading and release processes of coumarin-modified-insulin from the bilayer microcapsules.

The loading of the microcapsules with the different loads was evaluated by two methods:

(i) The concentration of the loads on the microparticles-in-microparticles was evaluated spectroscopically by determination of the absorbance of the loads in solution after and before deposition on the CaCO₃ microparticles-in-microparticles. This particles were used to assemble the microcapsules and the concentrations of the loads that were washed off upon the synthesis of the loaded microcapsules were added to the residual concentrations to yield a value of non-loaded substrates in the microcapsules. The difference between the initial added concentrations of the loads and the total concentrations of non-capsules-associated loads was assumed to be the loading degree of each of the loads within the microcapsules. (ii) The release process of the loads TMR-D, QDs or the labeled insulin from the microcapsules were recorded by subjecting the microcapsules to pH 5.5 (30 minutes), Zn²⁺-ions, 30mM (90 minutes) and glucose, 50mM (90 minutes). The recorded release profile for all loads reached saturation. For example, Figure S23 demonstrates the release profile of the coumarin-labeled-insulin. Knowing the concentrations of the microcapsules in each of the systems and applying appropriate calibration curves, the concentrations of the respective loads at the saturation levels of the release profiles were evaluated. Assuming that, the saturation levels in the release profiles corresponds to the total concentrations of the respective load in the carrier, this method provide an additional tool to estimate the degree of loading of the respective substrates. Indeed, the evaluated loading derived by this method overlap with 90%-100% of the loading degrees evaluated by method (i).
For example, from the saturated fluorescence intensity of the coumarin-labeled insulin and knowing the number of microcapsules we estimate the loading of insulin to be $1.3 \times 10^{-12}$ mole/microcapsule. Similarly, the loading of TMR-D and QDs corresponds to $1.8 \times 10^{-15}$ and $3.7 \times 10^{-14}$ mole/microcapsule, respectively.
Figure S24. Switchable release of insulin upon the treatment of the bilayer GOx/insulin loaded microcapsules-in-microcapsules with glucose, 7mM, followed by precipitation of the microcapsules, and their re-dispersion in a new buffer solution and switching on the release by re-added glucose, 7mM.
Figure S25. Schematic synthesis of stimuli-responsive microcapsules-in-microcapsules loaded with two different enzymes, GOx and Catalase, entrapped in the inner aqueous compartment, stabilized by a supramolecular duplex nucleic acid-bridged hydrogel, and the fluorophore-labeled insulin loaded in the outer aqueous compartment, stabilized by the pH-responsive hydrogel layer. The unlocking of the system by pH (glucose), results in the release of fluorophore-labeled insulin from the outer aqueous compartment.
Figure S26. Confocal microscopy images confirming the confinement of GOx, catalase and insulin into the respective aqueous containments. (A) Bright-field image of the bilayer hydrogel loaded microparticles. (B) FITC-GOx in the inner CaCO\textsubscript{3} core ($\lambda_{\text{ex}}$=490nm; $\lambda_{\text{em}}$ = 525nm). (C) Rhodamine-catalase in the inner CaCO\textsubscript{3} core ($\lambda_{\text{ex}}$=544nm; $\lambda_{\text{em}}$ = 576) (D) Coumarin-insulin in the outer CaCO\textsubscript{3} layer ($\lambda_{\text{ex}}$=360nm; $\lambda_{\text{em}}$ = 410nm). (E) Overlay of the different channels.
Figure S27. Calibration curves corresponding to (A) Tetramethylrhodamine-modified dextran, TMR-D (λ<sub>ex</sub> = 546 nm; λ<sub>em</sub> = 580 nm). (B) CdSe/ZnS QDs (2.25 nm) (λ<sub>ex</sub> = 464 nm; λ<sub>em</sub> = 482 nm). (C) Fluorescein isothiocyanate isomer I (FITC) (λ<sub>ex</sub> = 490 nm; λ<sub>em</sub> = 525 nm) (D) Rhodamine B isothiocyanate (Rhodamine) (λ<sub>ex</sub> = 544 nm; λ<sub>em</sub> = 576 nm). (E) 7-hydroxycoumarin-3-carboxylic acid N-succinimidyl ester (Coumarin) (λ<sub>ex</sub> = 360 nm; λ<sub>em</sub> = 410 nm).
Figure S28. Time-dependent fluorescence changes upon subjection the GOx and catalase/insulin bi-reservoir microcapsule-in-microcapsule system to glucose and analyze the GOx-generated H$_2$O$_2$ using the oxidation of amplex-red to the fluorescent resorufin as assay, curve (a). No fluorescence change is observed with the addition of catalase to the microcapsule-in-microcapsule system implying that the co-added catalase degraded any harmful peroxide. Time-dependent fluorescence change upon subjection the GOx/insulin bi-reservoir microcapsule-in-microcapsule system to glucose and analyze the GOx-generated H$_2$O$_2$ using the oxidation of amplex-red/resorufin assay, curve (b).
Evaluation of the molecular-weight cutoff value for the permeation of loads across single layer hydrogel microcapsules prepared by the integration of different loads in the microcapsules generated in the presence of $P_1$ and $P_2$.

The microcapsules were loaded with a series of FAM-modified nucleic acids of variable molecular weights $NA_1$-$NA_4$ (see Table S3). The release of the cargoes was examined in the stiffer hydrogel coating states and in the DNAzyme triggered lower stiffness coating states.

| Table S3: | Molecular Weight (KDa) | Sequence                                      |
|----------|------------------------|------------------------------------------------|
| $NA_1$   | 4.5                    | /56-FAM/TTAGGTAGTCG                           |
| $NA_2$   | 5.5                    | /56-FAM/TATATTAGGTAGG                        |
| $NA_3$   | 6.6                    | /56-FAM/TATAGCGAATTCTCAGCA                   |
| $NA_4$   | 8.1                    | TTTGGGTAGGGCGGTTGGTATA/36-FAM/               |

**Figure S29.** Fluorescence spectra corresponding to the release of respective loads from the microcapsules. The release of the fluorophore-labeled loads from the microcapsules was monitored in the stiffer hydrogel state (a) bridged by duplex $H_1/H_2/(x)$ and the $Zn^{2+}$-ion-dependent DNAzyme structure (3)/(4) and in the lower stiffness hydrogel state (b) generated upon the $Zn^{2+}$-ions triggered cleavage of structure (3)/(4).
Fluorescence spectra of the bulk solution were recorded after 60 min of interaction of the loaded microcapsule with a buffer solution. (A) - Release of \( \text{NA}_1 \) (B) - Release of \( \text{NA}_2 \) (C) - Release of \( \text{NA}_3 \) (D) - Release of \( \text{NA}_4 \).

The release process leads to the following conclusions:

\( \text{NA}_1 \)- Cannot be trapped in the higher- or lower-stiffness microcapsule states and it is washed out during the synthesis of the microcapsules.

\( \text{NA}_2 \)- Reveals partial release from the higher-stiffness microcapsules and free-release from the lower-stiffness microcapsules (the capsules reveal a lower loading degree due to the partial release of the load from the higher stiffness microcapsules during the synthesis of the microcapsules).

\( \text{NA}_3 \) and \( \text{NA}_4 \)- No release from the higher-stiffness microcapsules is observed. Release from the lower-stiffness microcapsules proceeds.
Table S4. Molecular weights and hydrodynamic radii corresponding to the different cargoes encapsulated in the bilayer microcapsules.

| Cargo    | Molecular weight [kDa] | Hydrodynamic radii |
|----------|------------------------|--------------------|
| TMR-D    | 70                     | 10.2±1.4 nm⁵       |
| Insulin  | 5.734                  | 1.5±0.1nm⁶         |
| GOx      | 160                    | 8.9 ± 0.4 nm⁷      |
| Catalase | 240                    | 9 nm⁸              |
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