Supporting Information

Bioinspired Theranostic Coordination Polymer Nanoparticles for Intranasal Dopamine-Replacement in Parkinson’s Disease

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Table of Content

ACRONYMS

MATERIAL AND METHODS

Materials .................................................................................................................. 4
HPLC Quantification of 1,4-Bis(imidazol-1-ylmethyl)benzene (BIX) in the DA-NCPs ........................................... 4
DA-content of DA-NCPs Determined by HPLC ........................................................................................................ 5
ICP-MS Quantification of Iron in DA-NCPs ........................................................................................................... 5
In vitro Release Kinetics Under Physiological Conditions .......................................................................................... 6
Processing and Post-Processing of MR Data ............................................................................................................ 6
Encapsulation of Epidermal Growth Factor (EGF) .................................................................................................. 8

SUPPORTING TABLES

Table S1. Experimental elemental analysis ............................................................................................................. 9
Table S2. Physical and conformational stability of DA-NCPs .................................................................................... 9
Table S3. IC50 values determined for free DA and DA-NCPs against HeLa and (BE)2-M17 cells ................................ 9
Table S4. In vitro r1 and r2 parameters and ex Vivo relative contrast enhancement .................................................. 9

SUPPORTING FIGURES

Figure S1. Dynamic light scattering (DLS) measurements for DA-NPs ................................................................. 10
Figure S2. Mössbauer spectra for DA-NCPs ................................................................................................................ 10
Figure S3. XRD pattern of DA-NCPs .......................................................................................................................... 11
Figure S4. FT-IR spectra ............................................................................................................................................. 11
Figure S5. 1H-NMR spectra .................................................................................................................................... 12
Figure S6. Calibration curve for DA concentration determination .............................................................................. 12
Figure S7. Encapsulation of recombinant EGF in DA-NCPs ...................................................................................... 13
Figure S8. In vitro release kinetics of dopamine from DA-NCPs .............................................................................. 13
Figure S9. Colloidal stability of DA-NCPs in human plasma ..................................................................................... 14
Figure S10. Effects of free DA and DA-NCPs on BE(2)-M17 dopaminergic cell survival ........................................ 14
Figure S11. Effects of DA and DA-NCPs on ROS production in BE(2)-M17 dopaminergic cells ......................... 15
Figure S12. Effects of iron (II) acetate on ROS production in BE(2)-M17 dopaminergic cells .............................. 15
Figure S13. Additional cytotoxicity experiments ...................................................................................................... 16
Figure S14. Extracellular DA levels determined by HPLC-ECD ............................................................................. 17
Figure S15. Evolution of the intracellular levels of the DA metabolites DOPAC and HVA ..................................... 18
Figure S16. Levels of DA, DOPAC, homovanilic acid (HVA), serotonin (5-HT) in the SNpc ................................ 19
Figure S17. T1 and T2 measurements ....................................................................................................................... 20
Figure S18. Intranasal administration of DA-NCPs ................................................................................................. 21

REFERENCES ......................................................................................................................................................... 22
ACRONYMS

AA: Ascorbic acid
BIX: 1,4-bis(imidazol-1-ylmethyl)benzene
BSA: Bovine Serum Albumin
CA: Contrast agent
Cb: Cerebellum
COMT: Catechol-O-methyltransferase
Ctx: Cortex
CytD: Cytochalasin D
DA: Dopamine or 3,4-dihydroxyphenethylamine
DA-NCPs: Dopamine Nanoscale Coordination Polymers
DATs: Dopamine transporters
DCF: Dichlorofluorescein
DCFDA: 2′,7′-Dichlorofluorescein diacetate
DHBA: Dihydroxybenzylamine
DLS: Dynamic Light Scattering
DOPAC: 3,4-dihydroxyphenylacetic acid
EDX: Energy-Dispersive X-Ray analysis
FBS: Fetal Bovine Serum
FT-IR: Fourier Transform Infrared Spectroscopy
GdDTPA: gadolinium diethylenetriaminepentacetate
HPLC: High-Performance Liquid Chromatography
HPLC-ECD: High-Performance Liquid Chromatography with Electrochemical Detection
HVA: Homovanilic acid
i.c.v: intracerebroventricular
i.v.: Intravenously
ICP-MS: Inductively Coupled Plasma-Mass Spectroscopy
L-DOPA: L-3,4-dihydroxyphenylalanine
LE: Loading Efficiency
MAO-B: Monoamine oxidase B
MAOB-I: Monoamine oxidase B inhibitor
MEM-α: Minimum Essential Medium α
MRI: Magnetic Resonance Imaging
MSA: Mouse Serum Albumin
MSME: Multi-Slice Multiecho Sequence
NCPs: Nanoscale Coordination Polymer
NM: Neuromelanin
NR: Number of Repetitions
NS-TEM: Negative-Staining Transmission Electron Microscopy
Nys: Nystatin
PBS: Phosphate-Buffered Saline
PD: Parkinson’s disease
PDA: Polydopamine
PDI: Polydispersity Index
PLGA: poly lactic-co-glycolic acid
PXRD: Powder X-Ray Diffraction
RARE: Rapid Acquisition with Relaxation Enhancement
RCE: Relative Contrast Enhancement
ROIs: Regions of Interest
ROS: Reactive Oxygen Species
SEM: Scanning Electron Microscopy
SNpc: Substantia Nigra pars compacta
Str: Striatum
TEM: Transmission Electron Microscopy
VTR: Variable Repetition Time
wt: Wild type
$^1$H-NMR: Proton Nuclear Magnetic Resonance
3-MT: 3-methoxytyramine
5HIIA: 5-hydroxyindoleacetic acid
5-HT: 5-hydroxytryptamine receptors
MATERIALS AND METHODS

Materials

All chemical reagents were purchased from Sigma-Aldrich (unless otherwise specified) and used as received. Solvents were obtained from Scharlab and used as received. Cell culture media and culture media supplements were ordered from Life Technologies. 1,4-Bis(imidazol-1-ylmethyl)benzene (BIX) was synthesized as previously reported. Self-assembled DA-based coordination polymer nanoparticles (DA-NCPs) were prepared under general aseptic conditions to ensure the suitability of the final material for biological experiments. Accordingly, the sterility of the final samples used for cell culture was evaluated following standard microbiological procedures. Cell lines were obtained from the American Type Culture Collection (ATCC).

HPLC Quantification of 1,4-Bis(imidazol-1-ylmethyl)benzene (BIX) in the DA-NCPs

The concentration of BIX present in the DA-NCPs was determined by HPLC-UV using an HPLC Waters 2695 separation module coupled to a Waters 2487 UV-Vis detector (suitable for dual detection). Analyses were performed using the Chromolith® Performance RP-18e (100 mm x 4.6 mm) column. Samples of DA-NCPs were prepared by dissolving 1.5 mg of dried nanoparticles in 0.15 mL of a methanol/HCL mixture (50 µl of HCl were dissolved in 1 mL methanol), and then sonicated for 20 min in an ultrasonic bath until a precipitate is observed. The initial samples were diluted to a final volume of 1.5 mL of Milli-Q water and centrifuged 10 min at 16000 x g. After centrifugation, the supernatants were further diluted in 0.1% (v/v) H₃PO₄ buffer containing 262 mg/L sodium 1-octanesulfonate. Before the analysis, the column was pre-equilibrated with 99% (v/v) buffer A (0.1% (v/v) H₃PO₄ buffer containing 262 mg/L sodium 1-octanesulfonate). Elution started with an isocratic gradient of 99 % (v/v) buffer A for
5 min, followed by gradual increase of buffer B (methanol absolute, HPLC grade) from 1% (v/v) to 40% (v/v) in 25 min. A calibration curve using BIX as external standard was prepared in triplicate. For this, standard solutions with 0, 10, 60, 110, 160 and 210 µg/mL of BIX were prepared by diluting a stock solution of BIX (960 µg/mL in a methanol/HCl mixture) in Milli-Q water. The methanol concentration of each standard was adjusted to reach a final water/methanol ratio of 90:10 (v/v). The elution of BIX was detected at 280 nm after 24 min chromatography. Results were adjusted to a linear regression model with R2>0.999 in the 10 to 210 µg/mL range.

**DA-content of DA-NCPs Determined by HPLC**

For DA quantification using HPLC, 5 mg of dried DA-NCPs were dissolved in 5 ml of homogenization solution containing 0.25 M perchloric acid, 100 µM sodium bisulphite, 250 µM EDTA. Samples were sonicated through an ultrasound bath until complete dissolution, further diluted in homogenization buffer up to three different concentrations, and subjected to HPLC-ECD analysis as described in the Methods section (see Simultaneous Determination of Monoamines using HPLC-ECD). The loading efficiency (LE) was determined by using Equation (1), as described for the UV-vis spectroscopic DA quantitation.

**ICP-MS Quantification of Iron in DA-NCPs**

The total iron present in DA-NCPs was determined by ICP-MS using a Perkin Elmer NexION 300X spectrometer equipped with an S10 autosampler. Samples of dried DA-NCPs nanoparticles were dissolved at a concentration of about 50 µg/mL in concentrated nitric acid solution and incubated at 25 ºC for 2 h. After incubation, samples were diluted (estimated concentration of iron ranging 10-200 ppb) in 0.5 % (v/v) HNO₃ and injected into the instrument using a peristaltic pump from 15 mL tubes placed in the autosampler. Calibration curves of
iron were prepared with five different concentrations (0, 10, 100, 200 and 500 ppb) by diluting certified reference metal ion solutions. The concentration of iron present in samples was calculated from the corresponding calibration curve, previously adjusted to a linear regression model ($R^2 > 0.99$).

**In vitro Release Kinetics Under Physiological Conditions**

*In vitro* release kinetics of DA from DA-NCPs was evaluated by the dialysis method. Briefly, a dialysis bag (MWCO: 3500 Da) containing DA-NCPs dispersed in sterile PBS was adjusted to pH 7.4 or pH 5.1 in the absence or presence of 0.5 mM BSA. To decrease dopamine oxidation during the experiment, all buffers were supplemented with 0.3 mg/mL of ascorbic acid (AA). Each dialysis bag was placed into 50 mL of buffer (pH 7.4 or pH 5.0) and incubated for 24 h at 37 ºC under constant stirring. To determine the release of dopamine from nanoparticles, and diffused through the dialysis bag, aliquots of 250 µL of the external solution were taken from the dialysate at different incubation times. Samples were diluted 50-fold in 1X homogenization solution (0.25 M perchloric acid, 100 µM sodium bisulphite, 250 µM EDTA), sonicated for 10 min, centrifuged 15 min at 16000 x g and stored at 4 ºC until analysis. Dopamine concentration in samples was determined by HPLC-ECD as previously described.

**Processing and Post-Processing of MR Data**

Processing and post-processing of $T_1$ and $T_2$ maps were carried out with Bruker software Paravision (version 5.1) by using the image sequence analysis (ISA) tool package. $T_1$ and $T_2$ weighted MRI were analyzed with ImageJ 1.49V (National Institutes of Health, USA).

Three regions of interest (ROIs) were manually defined after visual inspection both in the area of maximum enhancement and equivalent area of contralateral parenchyma. The relative contrast enhancement (RCE) - injection site ROI vs. contralateral parenchyma - obtained in
each case was used for calculations (see Equation S1 and S2). Only the slice with a better-defined contrast-enhanced region in the best study (clear detection of individual RCE regions) was used for measurements. Slices of similar anatomical positions were used for $T_1$ and $T_2$ RCE measurements.

$$RCE(\%)_{e_{x_{r}}o} = \left( \frac{S(i)}{S(c)} \right) \times 100$$

Equation (S1)

Where $S(i)$ is the absolute signal intensity of the “ipsilateral” region with respect to the contrast administration and that visually shows contrast enhancement, and $S(c)$ is the absolute signal intensity of the equivalent contralateral region, which serves as a control and that is defined as “100%”.

$T_1$ and $T_2$ values were estimated through different adjustments to map parameters using the following

$$y = A + C \times \left(1 - \exp\left(-t/T_1\right)\right)$$

Equation (S2)

where $y$ is the image signal intensity after $t$, $A$ is the absolute bias, $C$ is the estimated image signal intensity at $t = 0$ sec, and $t$ stands for the different TR used in the sequence.

$$y = A + C \times \exp\left(-t/T_2\right)$$

Equation (S3)

where $y$ is the image signal intensity after $t$, $A$ is the absolute bias, $C$ is the estimated image signal intensity at $t = 0$ sec, and $t$ stands for the different TE used in the sequence.

Finally, dual images reflecting both $T_1$ and $T_2$ RCE changes were generated with a post-processing algorithm of imaging division ($T_{1w}$ image / $T_{2w}$ image) producing a colored image highlighting dual RCE (figure 6c of the main manuscript).
Encapsulation of Epidermal Growth Factor (EGF)

To quantify the amount of encapsulated EGF, a series of dilutions of pure EGF were analysed by HPLC (Figure S7). The amount of loaded EGF has been calculated as the difference between the amount of EGF present in the initial reaction and the amount of protein determined from the supernatant after the synthesis. The equations used for calculation of the EE and LC are the following equations:

$$EE(\%) = \frac{\text{Weight of EGF in DA-NCPs}}{\text{Weight of initial EGF}}$$  \hspace{1cm} \text{Equation (S4)}

$$LC (\mu g/mg) = \frac{\text{Weight of EGF in DA-NCPs}}{\text{Total weight of DA-NCPs}}$$  \hspace{1cm} \text{Equation (S5)}
## SUPPORTING TABLES

### Table S1

**Table S1.** Experimental elemental analysis and calculated values for the formula [Fe(DA)_{1.6}(BIX)_{0.5}(AcO)_{0.2}(H_{2}O)_{1.9}]

|          | C %   | H %   | N %   | Fe % (ICP-MS) |
|----------|-------|-------|-------|---------------|
| Calculated | 43.71 | 5.00  | 8.78  | 10.02         |
| Experimental | 43.70 | 4.70  | 8.80  | 10.20         |

### Table S2

**Table S2.** Physical and conformational stability of DA-NCPs in different solvents and biocompatible buffers.

| Solvent/Buffer | Size (nm) \(^{[a]}\) | PDI     | ξ (mV) \(^{[b]}\) |
|---------------|----------------------|---------|-----------------|
| Water         | 171 ± 4.20           | 0.208 ± 0.022 | +25.2 ± 1.92   |
| PBS           | 1370 ± 721.0         | 0.484 ± 0.054 | -13.1 ± 0.53  |
| PBS-BSA       | 103 ± 16.2           | 0.198 ± 0.019 | -8.32 ± 0.25  |
| Culture medium | 107 ± 27.3           | 0.245 ± 0.011 | ND             |

\(^{[a]}\) Diameter of the nanoparticles determined by dynamic light scattering (DLS).  
\(^{[b]}\) Surface Z-potential. ND, not determined.

### Table S3

**Table S3.** IC50 values determined for free DA and DA-NCPs against HeLa and (BE)2-M17 cells.

| Cell type  | Treatment | IC50 ± SE (μg/mL) |
|------------|-----------|-------------------|
| (BE)2-M17  | Free Dopamine | 117 ± 11.8        |
|            | DA-NCPs    | 177 ± 36.1*       |
| HeLa       | Free Dopamine | 16 ± 6.6          |
|            | DA-NCPs    | 44 ± 10.9*        |

*\(^{p} < 0.05\), compared to the Free Dopamine control group; Statistical comparison of fits performed with GraphPad Prism software.

### Table S4

**Table S4.** In vitro \(r_1\) and \(r_2\) parameters and ex Vivo relative contrast enhancement (RCE for \(T_1\) and \(T_2\)) for the DA-NCPs and comparison with the commercial GdDTPA CA.

|          | In vivo phantoms | Ex vivo |
|----------|------------------|---------|
|          | Medium | \(r_1\) (mM s\(^{-1}\)) | \(r_2\) (mM s\(^{-1}\)) | \(r_2/r_1\) | Medium | RCE% (\(T_1\)) | RCE% (\(T_2\)) | RCE% (\(T_1/T_2\)) |
| DA-NCPs  | PBS    | 5.1 ± 0.6 | 1.4 ± 0.3 | 0.3       | PBS    | 262.0 ± 19.0 | 73.7 ± 12.0 | 3.6 ± 1.2       |
|          | +      | 4.4 ± 0.7 | 4.6 ± 0.2 | 1.1      | +      | 253.1 ± 32.8 | 74.9 ± 17.6 | 3.4 ± 1.4       |
|Vehicle   | Agarose| -        | -        | -        | BSA    | 118.6 ± 8.8 | 122.9 ± 11.5 | 0.97 ± 0.07    |
SUPPORTING FIGURES

Figure S1

81 ± 4 nm (PDI: 0.124)

Figure S1. Dynamic light scattering (DLS) measurements of DA-NCPs dispersed in ethanol. The average distribution size is shown with its corresponding deviation after three measurements.

Figure S2

Figure S2. Mössbauer spectra for DA-NCPs at 300 K. Experimental data (small blue dots), and computer fitted spectrum (big grey-black dots) for high-spin Fe(III). Hyperfine parameters of the fitting of the Mössbauer spectra at 300 K showed the isomer shift relative to the metallic iron (δFe), quadrupolar splitting (ΔEq) and the full width at half maximum (Γ). The spectrum was fitted to a single doublet with a ΔEq= 0.85 ± 0.04 mm/s and Γ= 0.28 mm/s. The fitting was centred at an isomeric shift δ= 0.41±0.02 mm/s attributed to high-spin Fe(III) ions.
Figure S3

Figure S3. XRD pattern of DA-NCPs

Figure S4

Figure S4. FT-IR spectra of BIX (red solid spectrum), dopamine (DA) (green solid spectrum) and DA-NCPs (blue solid spectrum). Main stretching (υ) and bending (δ) peaks are labeled to facilitate the comparison between spectra. Symbols are: Stretching band of aromatic ring (υC-Harom), stretching band of aliphatic chain (υC-Haliphatic), stretching band of hydroxyl group (υO-H), stretching band of amine group (υN-H), bending band of amine group (δN-H), stretching band of double bounds (υC=C), stretching band of catecholate group (υC-O).
**Figure S5.** $^1$H-NMR spectra of BIX (red solid spectrum), DA (green solid spectrum) and DA-NCPs (blue solid spectrum). Circles in the DA-NCPs spectrum show the presence of main contributing signals from BIX and DA, as expected.

**Figure S6.** Calibration curve for DA concentration determination using UV-vis spectroscopy.
Figure S7

**Figure S7**: Encapsulation of recombinant EGF in DA-NCPs. Representative HPLC-UV spectra of different EGF concentrations (10, 50 and 100 µg/mL). The supernatant (SN) of the reaction was analyzed for DA-NCPs prepared in the presence (DA-NCPs-EGF) or in the absence of EGF (DA-NCPs empty). For the synthesis of DA-NCPs, 75 µL of EGF at a concentration of 10 µg/µL were mixed with iron acetate solution, which was then quickly added to DA/BIX solution. The reaction was then followed as usual. As control condition, the same reaction was carried out by replacing the volume of EGF solution with PBS. The supernatants of each reaction was collected and lyophilized. These samples were suspended in 1 mL of 0.1 % v/v of trifluoroacetic acid (TFA) water solution, and a dilution of 1:4 in the same buffer was made. The diluted samples, along with different dilutions of EGF as standards, were analyzed by HPLC-UV using a C18 column.

Figure S8

**Figure S8.** *In vitro* release kinetics of dopamine from DA-NCPs. Experiments were performed in PBS containing 0.5 mM BSA at two different pH values of 7.4 (red dots) and 5.5 (blue squares).
Figure S9

Figure S9: Colloidal stability of DA-NCPs in human plasma. Dynamic light scattering (DLS) measurements of DA-NCPs dispersed in ethanol as reference sample and after incubation of the nanoparticles at 37ºC for different time points (0.5, 1, 1.5 or 6 h) with human plasma. Peak 1; shows the signal corresponding to DA-NCPs. Peak 2; indicates the signal attributable to plasma proteins.

Table S5. PDI values of DA-NCPs in ethanol or plasma at different time points.

| Solvent/Buffer | PDI       |
|----------------|-----------|
| Ethanol        | 0.150 ± 0.034 |
| Plasma 0.5 h   | 0.539 ± 0.011 |
| Plasma 1h      | 0.530 ± 0.021 |
| Plasma 1.5h    | 0.534 ± 0.007 |
| Plasma 6 h     | 0.519 ± 0.032 |

Figure S10

Figure S10. Effect of free DA and DA-NCPs on BE(2)-M17 dopaminergic cell survival. a) Dose-response effect of free DA and DA-NCPs on the viability of dopaminergic cells. To perform the assay, cells were incubated in the absence or the presence of different concentrations of DA (red bars) or DA-NCPs (green bars). After 48 h incubation, cell viability was determined by using a resazurin-based method (see Materials and Methods section for more details). Data are shown as mean ± SEM values (bars). At least three independent experiments were carried out for each concentration. Statistical analyses: ANOVA test was performed to compare DA and DA-NCPs treatments; *p<0.05 (n=6). b) Representative optical microscope images showing the effect of control medium (left), free DA (middle) or DA-NCPs (right) at a concentration of 200 µg/mL on the cells, after 48 h.
**Figure S11**

Effects of DA and DA-NCPs on ROS production in BE(2)-M17 dopaminergic cells. Cells were treated with: H₂O₂ as a positive control at a concentration of 250 µM (grey bars), free DA at three different concentrations (100 µg/mL, 10 µg/mL, and 1 µg/mL) and DA-NCPs with equivalent DA concentrations (green bars) for 24 h. Experiments were performed in the presence (+) or absence (-) of AA (20 µg/mL). In all the cases, values are mean ± SEM and n=2. *p < 0.05 compared to the control (CNT), and #p < 0.05, compared to the treatment without AA (one-way ANOVA and Tukey-HSD test).

**Figure S12**

Effects of iron (II) acetate on ROS production in BE(2)-M17 dopaminergic cells, after 24 h treatment at two different concentrations (30 and 3 µg/ml). H₂O₂ (250 µM) was used as a positive control. Treatments were carried out, in the absence (-) or presence (+) of AA (20 µg/mL). In all the cases, values are mean ± SEM and n=2. *p < 0.05 compared to the control (CNT), and #p < 0.05, compared to the treatment without AA (one-way ANOVA and Tukey-HSD test).
Figure S13. Additional cytotoxicity experiments. a) Effect of free DA and DA-NCPs on the viability of HeLa cells. b-d) Representative optical microscope images showing the effect of b) control medium, and of c) free DA or d) DA-NCPs at a concentration of 200 µg/mL on the cells after 48 h of treatment. In all plots, data are shown as mean ± SE values (bars). e-f) Dose-response effect of e) BIX and f) Fe\textsuperscript{II} acetate on the viability of dopaminergic cells at different concentrations ranging from 0 to 100 µg/mL. Representative optical microscopy images of cells incubated g) in the absence or the presence of h) BIX or i) Fe\textsuperscript{II} acetate at the highest concentration assayed (100 µg/mL).
**Figure S14.** Extracellular DA levels determined by HPLC-ECD after incubation of (BE)2-M17 cells for 2, 6 and 24 h with a) DA or b) DA-NCPs at two different concentrations (equivalent to 1 and 10 µg/mL of DA).
Figure S15. Evolution of the intracellular levels of the DA metabolites DOPAC and HVA over time in free DA- or DA-NCPs-treated cells. Levels measured upon cell treatment at 1 µg/mL of DA or with equivalent concentrations of the nanoparticles. b) Levels determined upon incubation with 10 µg/mL of DA or with equivalent concentrations of DA-NCPs. Values are mean ± SEM. Statistical analysis: three-way ANOVA (full factorial) and Sidak post-hoc correction was applied to the log-transformed data. *p < 0.05, compared to basal levels (0 µg/mL); #p < 0.05, compared to treatment with DA; a p < 0.05, compared to the levels found after 2 h; b p < 0.05, compared to levels found after 6 h;
Figure S16. Levels of DA (DA), DOPAC (dopac), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the SNpc in the right (panels a, c, e, g and i) and left (panels b, d, f, h and j) brain hemispheres of adult Sprague-Dawley rats administered i.c.v with the DA-NCPs. The nanoparticles were infused in the right ventricle, as shown in Figure 5a. The levels of the different catecholamines were determined by HPLC-ECD at different time points (2, 6 and 24 h) post-DA-NCPs injection. Values are given as mean ± SEM and n=3 independent experiments unless otherwise specified.
**Figure S17.** $T_1$ and $T_2$ measurements for a) DA-NCPs and b) commercial Gd-based contrast agent (GdDTPA, Magnevist®). Data are shown as mean ± SEM. The experimental data were linearly fitted to obtain the slope of each line (quantitative determination of $r_1$ and $r_2$). The results for the DA-NCPs synthesized are shown. From the relaxation times ($T_1$ and $T_2$), the relaxivities parameters can be calculated as follow: *Relaxation times:* $T_{1,2}$ [s]; *Relaxation parameters:* $R_{1,2}$ [s$^{-1}$]; *Representation $R_{1,2}$ vs [metal];* $R_{1,2}/[\text{metal}] = r_{1,2}$ (relaxivity parameters).
Figure S18

Intranasal administration of DA-NCPs. a) Longitudinal and b) coronal sections of in vivo MRI showing the nasal cavity of a rat after (post-treatment) the intranasal application of the nanoparticles. c) Levels of DA in the left striatum of adult Sprague-Dawley rats administered intranasally with vehicle (light pink) or DA-NCPs (green). The red line in b), demarcates the regions of interest (ROIs) used for the analyses.
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