Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Stimuli-responsive Local Drug Molecule Delivery to Adhered Cells in a 3D Nanocomposite Scaffold

Andisheh Motealleh,[a] *Rossella De Marco,*[b] *and Nermin Seda Kehr**[a]

[a]Physikalisches Institut and Center for Soft Nanoscience,Westfälische Wilhelms-Universität Münster, Busso-Peus Straße 10, D-48149 Münster, Germany

[b]Department of Chemistry "G. Ciamician,", University of Bologna, via Selmi 2, 40126 Bologna, Italy

Synthesis of PMO-NH_2: To synthesize the PMOs, 484.5 mg of CTAB was dissolved in 90 mL of H_2O, 33 mL of ethanol, and a 28 wt% ammonia (0.075 g) solution. The reaction mixture was stirred at room temperature for 1 h before the addition of BTME (1.27 g). The above reaction mixture was continuously stirred for an additional 48 h at room temperature. The CTAB mesoporous template was removed by stirring the sample in ethanol (50 mL) containing a 36 wt% aqueous solution of HCl (1.5 g) at 50 ^oC for 6 h. The resulting solid (PMO) was recovered by centrifugation, washed with ethanol several times, and dried at 60 ^oC in a vacuum. Then, PMO (100 mg) was reacted with 3-aminopropyltrimethoxysilane (APTES) (0.8 mL) in toluene (5 mL) at 60 ^oC for 18 h. The final product (PMO-NH_2) was recovered by centrifugation, washed with ethanol several times, and dried at 60 ^oC in a vacuum.

Determination of the amount of DOX in ^{DOX}PMO-NH_2 (and later, ^{DOX}PMO-(PSS)PLL: Fluorescence spectrophotometry was used to quantitatively determine the amount of DOX in ^{DOX}PMO-NH_2 and ^{DOX}PMO-(PSS)PLL. First, to produce the calibration curve for DOX, different concentrations of DOX were suspended in cell culture media. The emission spectra of the different DOX concentrations were measured between 500 nm and 700 nm. The emissions at λ_{max}= 550 nm were plotted as a function of the DOX concentration, and the respective slope was evaluated. The relationship y = 20224x + 10474 was obtained from the plotted graph (y is emission and x is the concentration of DOX). Then, to assess the amount of DOX loaded into the PMOs, a suspension of 1 mg of ^{DOX}PMO-NH_2 (as well as 1 mg of ^{DOX}PMO-(PSS)PLL)) was prepared in 2 mL cell culture media and kept on a stirrer for 20 min, then the emission spectra of these suspensions were measured. The amount of DOX in the ^{DOX}PMO-NH_2 (as well as ^{DOX}PMO-(PSS)PLL) was determined using the formula obtained from the DOX calibration curve.

Furthermore, the drug encapsulation efficiency (EE) and drug loading capacity (LC) were calculated using the following equations, respectively.

$$\%EE = [(\text{Drug added} - \text{Free drug})/\text{Drug added}] * 100 \quad (1)$$

$$\%LC = [\text{Entrapped Drug/nanoparticles weight}] * 100 \quad (2)$$

Coating the PMO-NH_2 and ^{DOX}PMO-NH_2 with PSS and then PLL [^{DOX}PMO-(PSS)PLL]: To functionalize the PMO-NH_2 and ^{DOX}PMO-NH_2 particles, we first coated them with PSS, followed by coating them with the biopolymer PLL. For PSS coating, PMO-NH_2/^{DOX}PMO-NH_2 (40 mg) was suspended in 1 mL water, mixed with PSS (MW: 70,000) (0.5 mg), and sonicated for 10 min. This reaction mixture was stirred overnight at room temperature. After the reaction, coated PMOs were washed several times with water to remove unreacted PSS and dried at room temperature. After PSS coating, PMOs were coated with PLL (MW:15,000-30,000) (0.5 mg/mL water) using the same procedure, and the coating process was applied two more times by alternating PSS and PLL. The final products PMO-(PSS)PLL and ^{DOX}PMO-(PSS)PLL were obtained by centrifugation, washed with water 2× and dried at room temperature.

Determination of the amount of PLL and PSS on PMO-(PSS)PLL and ^{DOX}PMO-(PSS)PLL: Ultraviolet–visible **(**UV/Vis) spectrophotometry was used to quantitatively determine the amount of PLL and PSS on PMO-(PSS)PLL/^{DOX}PMO-(PSS)PLL. First, different concentrations of PLL and PSS were suspended in water. The absorption spectra of these suspensions were measured against water between 190 nm and 300 nm for PLL and 200 nm and 800 nm for PSS using a quartz cell with a path length of 1 cm. The absorbance at λ_{max}= 210 nm and λ_{max}= 226 nm was plotted as a function of the PLL and PSS concentration, respectively, and

the slopes of these curves were evaluated. The formulas of y = 2.0257x + 0.2131 and y = 3.3181x + 0.0154 were obtained from the plotted graphs of PLL and PSS, respectively (y is the absorbance and x is the concentration of PLL and PSS).

Once calibration curves were established, solutions of PLL and PSS (ca. 0.5 mg) in water (1 mL) were prepared, and the absorption spectra of these solutions were measured. The amount of PLL and PSS in water was determined using the formula associated with the PLL and PSS graphs; these values were called $C1_{PLL}$ and $C1_{PSS}$. Thereafter, PMO-NH_2/^{DOX}PMO-NH_2 (40 mg) was suspended first in the water solution containing PSS. The reaction mixtures were stirred for 1 day at room temperature. The PSS-coated PMOs were removed from the supernatant by centrifugation, washed with water 2×, centrifuged, and all the supernatant in each step was collected. The absorption spectrum of the supernatant was measured against water between 200 nm and 800 nm using a quartz cell with a path length of 1 cm. The amount of PSS in the supernatant was determined using the formula of the plotted graph of PSS; this value was called as $C2_{PSS}$. Then, we determined the amount of PSS (in the 1st layer) that had adsorbed onto ^{DOX}PMO-NH_2; this value was called $C3_{PSS}$ and was calculated using formula below. We calculated the amounts of PLL (in the 2nd and 4th layers) and PSS (in 3th layer) that had adsorbed onto the respective PSS-coated PMOs using the same procedure and similar formulas (3, 4).

$$C3_{PSS} = C1_{PSS} - C2_{PSS} \quad (3)$$

$$C3_{PLL} = C1_{PLL} - C2_{PLL} \quad (4)$$

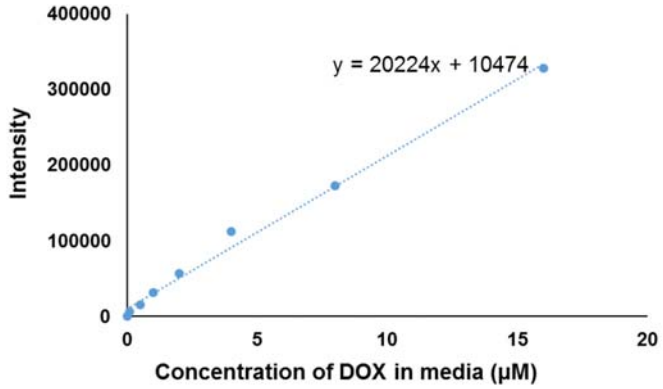

Figure S1. The calibration curve of the DOX which was obtain by mixing different concentration of DOX in cell culture media.

Table S1. Quantitative amount of free PSS/PLL (μg) in water (1 ml) after each centrifugation (for 1 mg of the respective PMOs).

	1st Layer PSS	2nd Layer PLL	3th Layer PSS	4th Layer PLL
PMO-(PSS)PLL	1.212 ± 0.003	1.465 ± 0.002	1.125 ± 0.004	1.123 ± 0.006
^{DOX}PMO-(PSS)PLL	1.158 ± 0.003	1.775 ± 0.006	1.145 ± 0.002	1.112 ± 0.003

Table S2. The quantitative amount of DOX (μg) in ^{DOX}PMO-NH_2 and ^{DOX}PMO-(PSS)PLL (1 mg).

	^{DOX}PMO-NH_2	^{DOX}PMO-(PSS)PLL
DOX	44.3 ± 0.2	43.8 ± 0.2

Table S3. DLS of PMO-(PSS)PLL, ^{DOX}PMO-NH_2 and ^{DOX}PMO-(PSS)PLL.

	Diameter (DLS, nm) Water
PMO-(PSS)PLL	425.6 ± 18.2
^{DOX}PMO-(PSS)PLL	440.4 ± 6.1
^{DOX}PMO-NH_2	299.6 ± 28.7

Table S4. Zeta potential value of PMO-NH_2 and ^{DOX}PMO-NH_2 with PSS and PLL.

coating layer	**ZP, mV**
PMO-NH_2	32.1 ± 0.1
1st layer (PSS)	-48.0 ± 1.4
2nd layer (PLL)	20.9 ± 1.9
3th layer (PSS)	-40.1 ± 0.4
4th layer (PLL)	29.3 ± 0.6
^{DOX}PMO-NH_2	31.0 ± 1.2
1st layer (PSS)	-43.1 ± 1.1
2nd layer (PLL)	28.4 ± 0.1
3th layer (PSS)	-43.3 ± 1.5
4th layer (PLL)	26.5 ± 0.8

Table S5. ^{DOX}PMO-NH_2 and ^{DOX}PMO-(PSS)/PLL distribution on 1 μm^2 area of Alg, [the number of repeated experiments (N) = 3].

	Number of PMOs on 1 μm^2 area of Alg
^{DOX}PMO-NH_2	12.2 ± 2.4
^{DOX}PMO-(PSS)/PLL	11.9 ± 3.7

Table S6. Quantitative amount of ^{DOX}PMO-NH_2 and ^{DOX}PMO-(PSS)/PLL in 1 mL NC hydrogels, [the number of repeated experiments (N) = 3].

	w/v %
^{DOX}PMO-NH_2	0.217 ± 0.008
^{DOX}PMO-(PSS)/PLL	0.223 ± 0.007

Table S7. Swelling Ratio of alginate and NC scaffolds after 1 day and 4 and 7 days incubation at 37°C, [the number of repeated experiments (N) = 3].

	Alg	**^{Dox}PMO-NH_2-Alg**	**^{DOX}PMO-(PSS)/PLL-Alg**
1 day	34.7 ± 6.9	56.7 ± 9.3	59.2 ± 7.6
3 days	35.4 ± 5.7	57.6 ± 9.6	59.9 ± 8.9
7 days	34.3 ± 6.3	56.4 ± 7.5	58.1 ± 8.8

Table S8. Weight loss (%) of alginate and NC scaffolds after 1 day and 4 and 7 days incubation in presence of cells at 37°C, [the number of repeated experiments (N) = 3].

Fibroblast cells in scaffold	**Alg**	**^{Dox}PMO-NH_2-Alg**	**^{DOX}PMO-(PSS)/PLL-Alg**
1 day	0.46 ± 0.03	0.17 ± 0.002	0.14 ± 0.04
3 days	0.61 ± 0.04	0.23 ± 0.02	0.22 ± 0.03
7 days	0.69 ± 0.06	0.28 ± 0.02	0.25 ± 0.02
Colo 818 cells in scaffold			
1 day	0.52 ± 0.02	0.22 ± 0.03	0.19 ± 0.05
3 days	0.74 ± 0.07	0.29 ± 0.06	0.28 ± 0.01
7 days	0.84 ± 0.03	0.35 ± 0.05	0.33 ± 0.05

Table S9. Porosity (%) and pore size (μm) of alginate and NC alginate scaffolds.

	Alg	**^{DOX}PMO-NH_2-Alg**	**^{DOX}PMO-(PSS)/PLL-Alg**
Porosity	42.5 ± 2.4	49.1 ± 2.2	56.2 ± 5.1
Pore size	345.5 ± 23.4	254.31 ± 15.2	252.2 ± 12.1

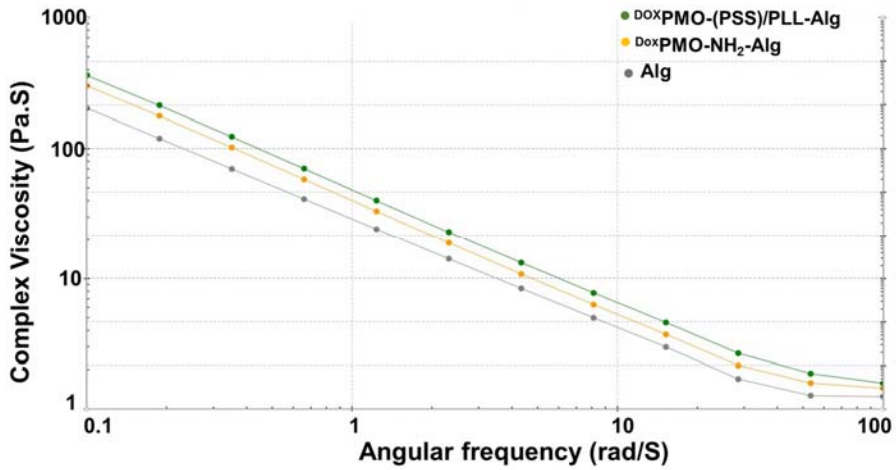

Figure S2. Viscosity values as a function of angular frequency for pure Alg, ^{DOX}PMO-NH_2-Alg, and ^{DOX}PMO-(PSS)PLL-Alg hydrogel.

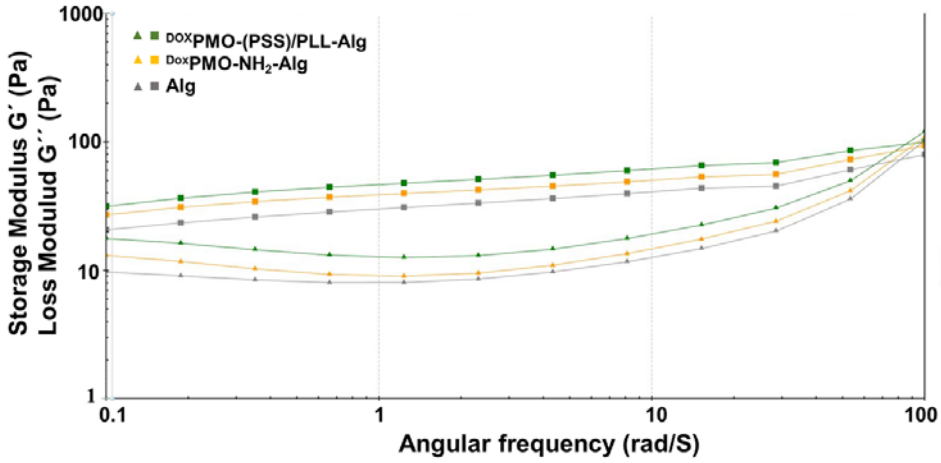

Figure S3. The dependence of storage modulus (G´, the square symbols) and loss modulus (G´´, the triangle symbols) with angular frequency for pure Alg, ^{DOX}PMO-NH_2-Alg, and ^{DOX}PMO-(PSS)PLL-Alg hydrogel.

Table S10. The amount of DOX (µg/ml) released from ^{DOX}PMO-NH_2-Alg and ^{DOX}PMO-(PSS)/PLL-Alg over different time intervals at pH 7.4 and pH 6.0 (N=6).

	^{Dox}PMO-NH_2-Alg pH 6.0	**^{Dox}PMO-NH_2-Alg pH 7.4**	**^{Dox}PMO-(PSS)/PLL-Alg pH 6.0**	**^{Dox}PMO-(PSS)/PLL-Alg pH 7.4**
3 min	2.91 ± 0.03	1.19 ± 0.02	1.34 ± 0.05	0.81 ± 0.03
15 min	5.12 ± 0.05	2.43 ± 0.04	2.88 ± 0.08	1.70 ± 0.59
30 min	7.17 ± 0.09	3.42 ± 0.05	4.48 ± 0.22	2.49 ± 0.84
1 h	8.45 ± 0.11	4.51 ± 0.07	6.04 ± 0.28	3.14 ± 0.86
2 h	9.84 ± 0.18	5.64 ± 0.09	7.69 ± 0.34	4.08 ± 0.88
3 h	11.29 ± 0.21	7.02 ± 0.15	9.28 ± 0.36	4.84 ± 1.17
4 h	12.76 ± 0.27	8.61 ± 0.17	10.89 ± 0.41	5.66 ± 1.46
5 h	14.14 ± 0.30	10.15± 0.21	12.44 ± 0.41	6.44 ± 1.69
7 h	15.20 ±0.31	11.36 ± 0.23	13.99 ± 0.44	7.12 ± 1.71
1 day	16.68 ± 0.34	12.77 ± 0.27	15.49 ± 0.48	8.65 ± 1.72
2 days	18.96 ± 0.59	13.94 ± 0.31	16.81 ± 0.52	9.59 ± 1.73
3 days	20.94 ± 0.61	15.36 ± 0.34	18.07 ± 0.57	10.56 ± 1.75
7 days	23.62 ± 0.63	16.42 ± 0.38	19.43 ± 0.59	11.35 ± 1.77
14 days	27.08 ± 0.63	17.29 ± 0.40	20.86 ± 0.63	12.22 ± 1.86
30 days	29.43 ± 0.69	18.82 ± 0.41	21.85 ± 0.68	12.96 ± 1.90

Table S11. The released % amount of DOX from ^{DOX}PMO-NH_2-Alg and ^{DOX}PMO-(PSS)/PLL-Alg in different time periods.

	^{Dox}PMO-NH_2-Alg pH 6.0	**^{Dox}PMO-NH_2-Alg pH 7.4**	**^{Dox}PMO-(PSS)/PLL-Alg pH 6.0**	**^{Dox}PMO-(PSS)/PLL-Alg pH 7.4**
3 min	7 (%) ± 2.2	3 (%) ± 1.6	3 (%) ± 0.5	2 (%)± 0.3
15 min	11 (%) ± 2.1	5 (%) ± 1.6	7 (%) ± 2.5	4 (%) ± 0.3
30 min	16 (%) ± 3.4	8 (%) ± 1.3	10 (%) ± 1.4	6 (%) ± 0.2
1 h	19 (%) ± 2.2	10 (%) ± 1.8	14 (%) ± 3.4	7 (%) ± 1.5
2 h	22 (%) ± 7.3	13 (%) ± 2.1	17 (%) ± 4.4	9 (%) ± 2.1
3 h	25 (%) ± 3.1	16 (%) ± 5.7	21 (%) ± 1.4	11 (%) ± 2.9
4 h	29 (%) ± 5.9	19 (%) ± 2.4	25 (%)± 5.1	13 (%) ± 3.1
5 h	32 (%) ± 2.7	23 (%) ± 3.4	28 (%)± 0.6	15 (%) ± 2.2
7 h	34 (%) ± 1.3	26 (%) ± 1.8	32 (%)± 2.6	16 (%) ± 1.8
1 day	38 (%) ± 2.6	29 (%) ± 4.6	35 (%)± 4.2	20 (%)± 1.5
2 days	43 (%) ± 2.7	31 (%)± 4.0	38 (%)± 4.3	22 (%)± 1.1
3 days	47 (%) ± 1.9	35 (%)± 3.0	41 (%)± 4.7	24 (%)± 1.8
7 days	53 (%) ± 1.5	37 (%)± 3.5	44 (%)± 2.1	26 (%)± 1.5
14 days	61 (%) ± 1.6	39 (%)± 2.3	48 (%)± 4.1	28 (%)± 2.5
30 days	66 (%)± 5.4	42 (%)± 1.1	50 (%)± 4.6	29 (%)± 2.9

Table S12. The amount of alive fibroblasts and Colo 818 cells(x 10^3) and cell viability (in parenthesis) into PMO-(PSS)/PDL-Alg, ^{DOX}PMO-NH_2-Alg, and ^{DOX}PMO-(PSS)/PLL-Alg at different incubation times (N=6).

	Fibroblast alive cells			**Colo 818 alive cells**		
	PMO-(PSS)/PLL-Alg	**^{Dox}PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-NH_2-Alg**	**PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-NH_2-Alg**
30 min	18.5 ± 0.8 (93%)	16.5 ± 0.9 (81%)	14.9 ± 0.6 (75%)	17.6 ± 0.5 (84%)	14.8 ± 0.5 (76%)	11.8 ± 0.5 (60%)
2 h	18.1 ± 0.8 (90%)	16.6 ± 0.9 (79%)	14.6 ± 0.7 (71%)	17.3 ± 0.5 (83%)	15.3 ± 0.5 (72%)	11.0 ± 0.8 (55%)
4 h	18.6 ± 0.9 (88%)	17.1 ± 0.6 (74%)	14.3 ± 0.9 (67%)	17.8 ± 0.5 (81%)	15.5 ± 0.6 (60%)	9.8 ± 0.5 (50%)
7 h	19.0 ± 0.8 (86%)	17.9 ± 0.8 (73%)	13.4 ± 0.7 (64%)	18.3 ± 0.5 (80%)	15.8 ± 0.5 (58%)	9.5 ± 0.6 (45%)
1 day	20.9 ± 0.6 (82%)	18.9 ± 0.8 (71%)	12.5 ± 0.9 (57%)	19.5 ± 0.6 (79%)	16.3 ± 0.5 (56%)	8.3 ± 0.5 (38%)
2 days	22.3 ± 1.0 (82%)	20.4 ± 1.2 (70%)	11.8 ± 1.0 (55%)	20.5 ± 0.6 (78%)	17.3 ± 0.5 (55%)	8.0 ± 0.8 (36%)
3 days	22.5 ± 1.2 (80%)	21.3 ± 1.0 (66%)	11.4 ± 0.5 (52%)	21.5 ± 0.6 (72%)	18.3 ± 0.5 (54%)	7.8 ± 0.5 (35%)
7 days	28.0 ± 0.8 (78%)	25.6 ± 1.3 (63%)	10.6 ± 0.9 (39%)	26.3 ± 0.5 (71%)	23.5 ± 0.6 (51%)	6.8 ± 0.5 (25%)
14 days	28.25 ± 0.7 (67%)	22.8 ± 1.2 (54%)	6.4 ± 0.7 (25%)	26.5 ± 0.6 (63%)	18.8 ± 0.5 (42%)	3.8 ± 0.5 (14%)

Table S13. The alive cell amount ($x10^3$) of fibroblast and Colo 818 cells in PMO-(PSS)/PLL-Alg, ^{DOX}PMO-(PSS)/PLL-Alg and ^{DOX}PMO-NH_2-Alg in different incubation time periods determined by presto blue assay.

	Fibroblast alive cells			**Colo 818 alive cells**		
	PMO-(PSS)/PLL-Alg	**^{Dox}PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-NH_2-Alg**	**PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-NH_2-Alg**
2 h	20.5 ± 3.6	20.3 ± 2.2	19.7 ± 3.5	19.6 ± 5.7	19.2 ± 5.0	18.9 ± 6.2
1 day	22.3 ± 3.3	19.4 ± 5.4	13.4 ± 4.6	20.4 ± 5.0	18.9 ± 5.5	11.8 ± 4.2
3 days	25.1 ± 4.0	23.3 ± 5.8	11.6 ± 2.9	24.1 ± 5.7	21.5 ± 2.8	9.6 ± 2.6
7 days	27.5 ± 5.2	24.4 ± 2.4	8.4 ± 2.3	26.3 ± 4.2	21.8 ± 4.6	7.0 ± 2.7

Table S14. Cell viability (%) determined by Prestoblue assay and Hemocytometer.

	Cell viability Fibroblast cells in scaffold			**Cell viability Colo 818 Cells in scaffold**		
	PMO-(PSS)/PLL-Alg	**^{Dox}PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-NH_2-Alg**	**PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-(PSS)/PLL-Alg**	**^{Dox}PMO-NH_2-Alg**
2 h	95%	85%	77%	92%	77%	69%
1 day	86%	72%	55%	81%	64%	44%
3 days	80%	70%	47%	73%	59%	35%
7 days	79%	65%	31%	72%	56%	23%