

Alginate Microsponges as a Scaffold for Delivery of a Therapeutic Peptide against Rheumatoid Arthritis

Section S1. Determination of the NaAlg content in the microsponges

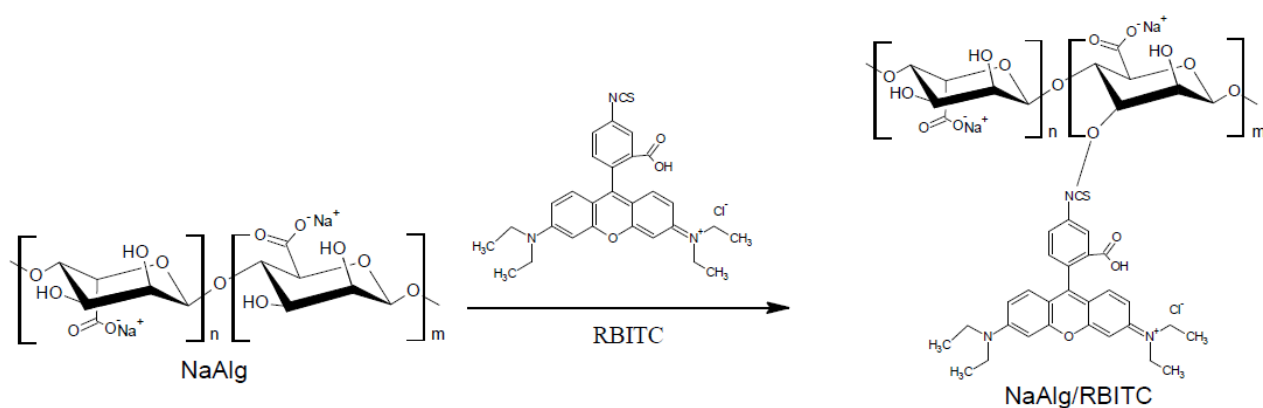


Figure S1. Reaction scheme of NaAlg with Rhodamine B Isotiocyanate.

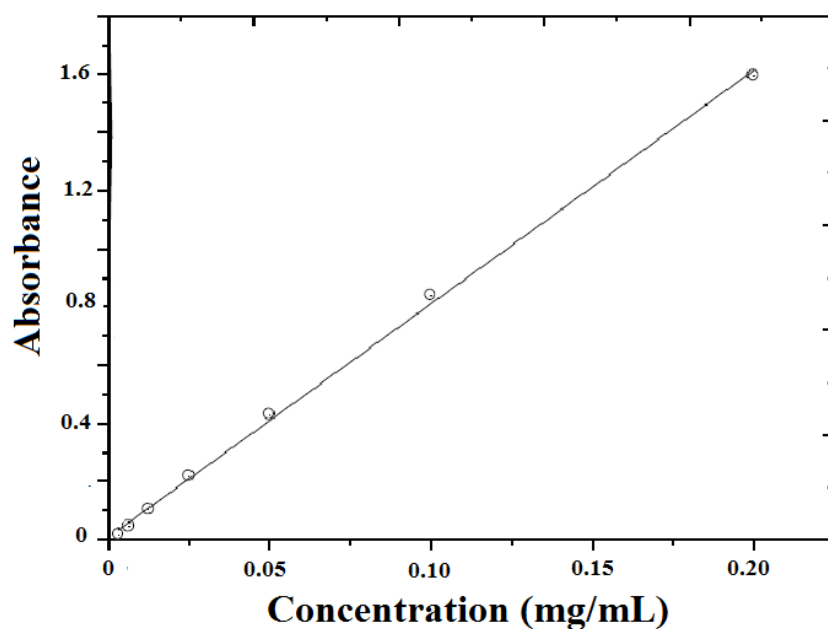


Figure S2. Calibration curve of RBITC in aqueous solution obtained by UV-Vis absorption measurements ($\lambda=545$ nm).

Section S2. Viscosity measurements

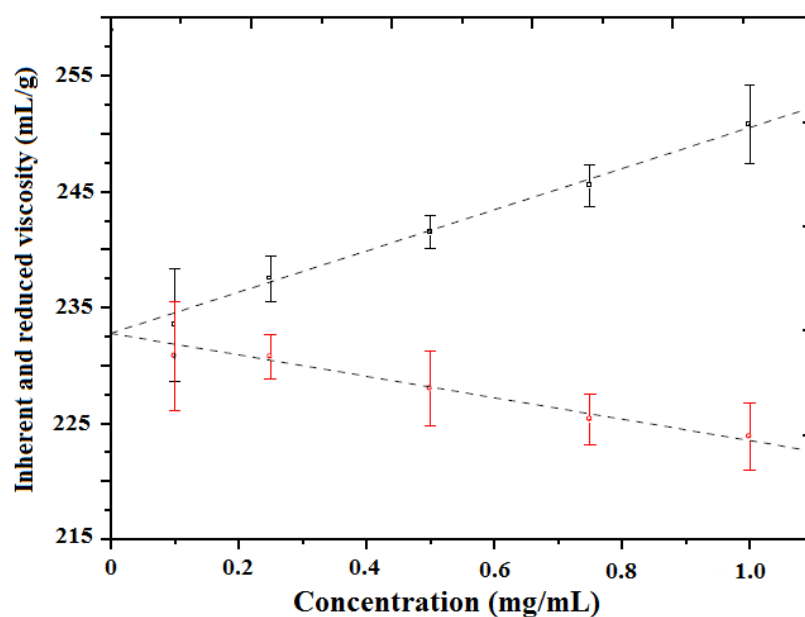


Figure S3. Viscosity measurements of NaAlg solutions in 0.1 M NaCl. Black dots: reduced viscosities; red dots: inherent viscosities. Experimental reduced and inherent viscosities (Table T1) were fitted through the Mark-Houwink-Sakurada and Huggins-Kraemer equations, respectively (dotted lines).

Table S1. Relative, specific, reduced and inherent viscosities for different concentrations of NaAlg solutions in 0.1 M NaCl (T=25°C).

<i>NaAlg</i> Concentration (mg/mL)	η_{rel} (mL/g) $\pm SD$	η_{sp} (mL/g) $\pm SD$	η_{rid} (mL/g) $\pm SD$	η_{inh} (mL/g) $\pm SD$	$[\eta]$ (mL/g) $\pm SD$	<i>PM</i> (kDa) $\pm SD$
0.10	1.04 ± 0.01	0.04 ± 0.01	233 ± 5	231 ± 5		
0.25	1.11 ± 0.01	0.11 ± 0.01	237 ± 2	231 ± 2		
0.50	1.22 ± 0.01	0.22 ± 0.01	241 ± 1	228 ± 3	233 ± 3	78.56 ± 0.01
0.75	1.35 ± 0.02	0.35 ± 0.02	245 ± 2	225 ± 2		
1.00	1.49 ± 0.02	0.49 ± 0.02	251 ± 3	224 ± 3		

Section S3. Fluorescence of RITBC in aqueous solution and in the supernatant solution.

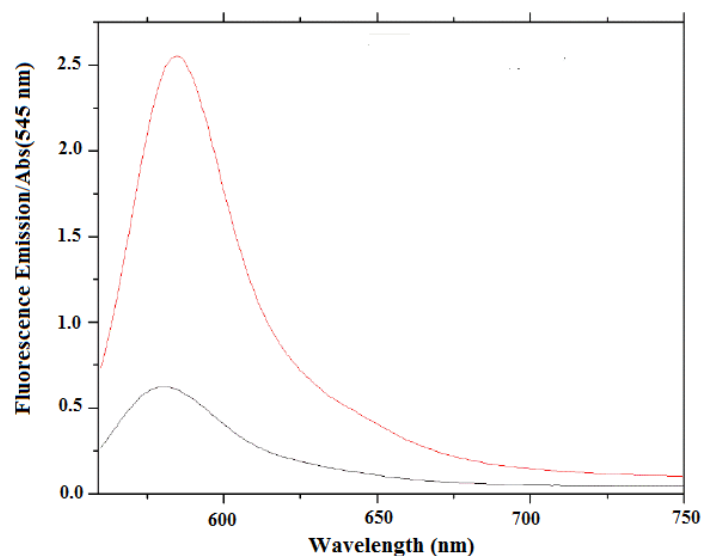


Figure S4. Emission spectra ($\lambda_{\text{ex}}=545$ nm) of RBITC/NaAlg in aqueous solution (light-blue) and as supernatant (red).

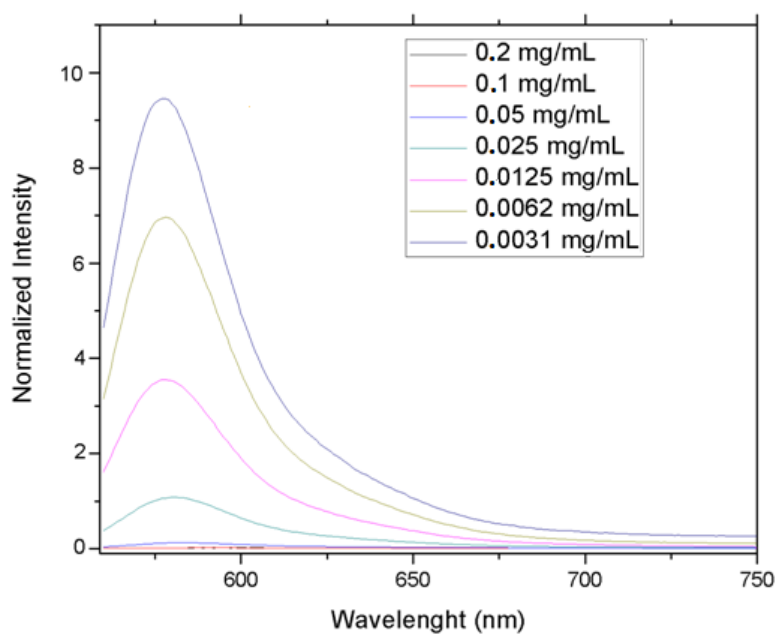


Figure S5. Fluorescence spectra of RITBC in aqueous solution at different concentrations

Section S4. Imaging of NaAlg PMS

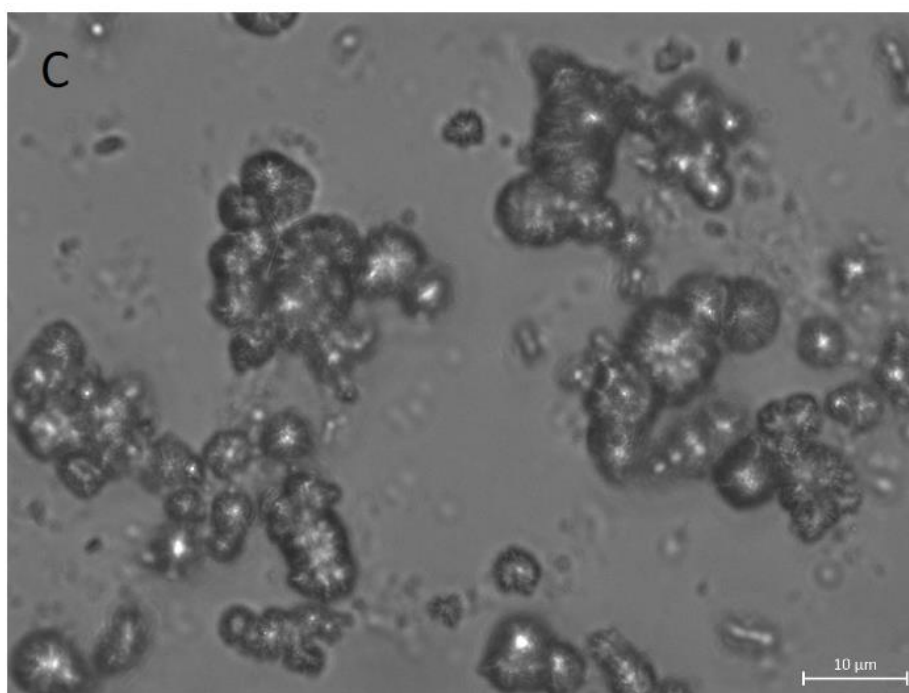


Figure S6. Optical microscopy image (100x) of microparticles obtained by aggregation of the cross-linker molecules (Cys+CDI).

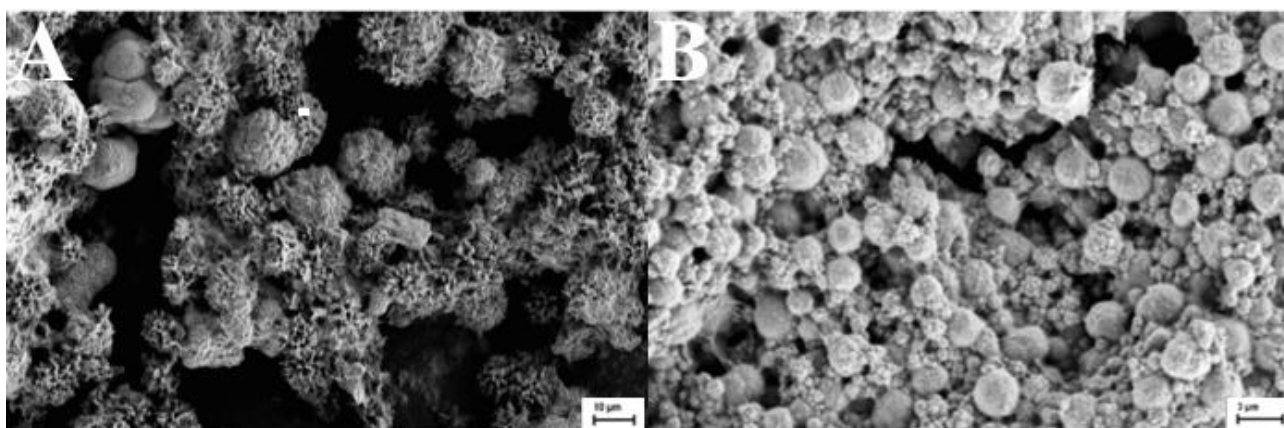


Fig. S7. SEM image of microstructures formed by : A) CL molecules (scale bar 10 μm); B) NaAlg-CL polymer (scale bar: 3 μm).

Section S5. Determination of the size distribution of alginate microsponges

Confocal Laser Scanning Fluorescence Microscopy

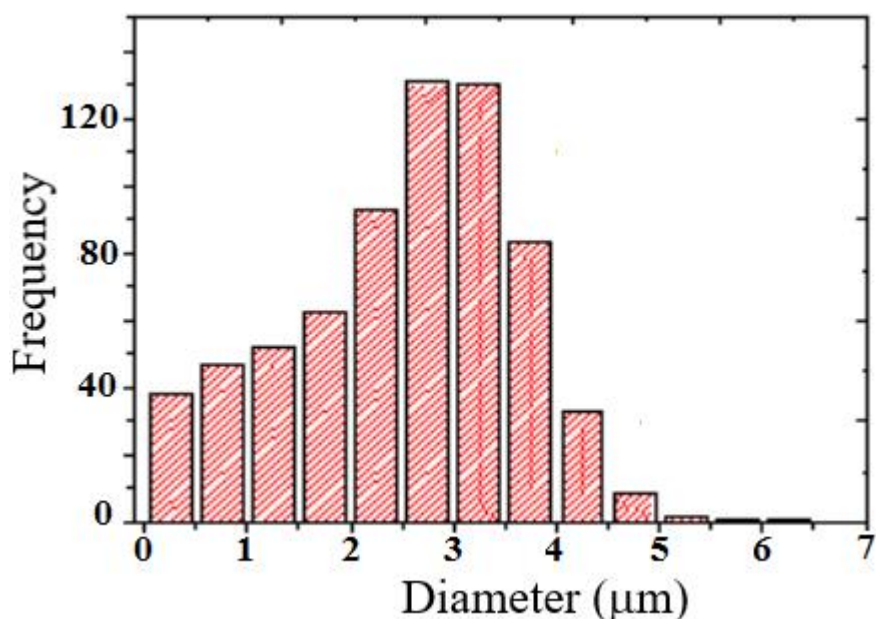


Fig. S8. Size distribution (frequency) of NaAlg PMS. The polymer was functionalised by rhodamine B (RBITC). The histograms were obtained by software analysis (ImageJ) of fluorescence confocal microscopy images.

DoE analysis of DLS experiments

Let us consider the PMS diameter (μm), as provided by DLS experiments, the output parameter Y , and the Cys and CDI weights (mg), and H_2O volumes (μL) the input variables of the process. Output and input variables are linked by the functional relation:

$$\text{diameter} = f(\text{Cys}, \text{CDI}, \text{H}_2\text{O})$$

To the aim we applied a 2^3 , *i.e.*, 2 values for each variable, regression model (Table S2).

Table S2. Input parameters (X) of DoE analysis

	<i>Parameter</i>	<i>Unit</i>	<i>Low level</i> <i>(-I)</i>	<i>High level</i> <i>(+I)</i>
X_1	Cys	mg	20	30
X_2	CDI	mg	20	30
X_3	H_2O	μL	200	300

A 2^3 DoE analysis requires 8 experiments and 7 parameters. Three parameters are associated to the {Cys, CDI, H_2O } concentrations, and four to the {Cys · CDI, Cys · H_2O , CDI · H_2O , Cys · CDI · H_2O } interactions. As all experiments were carried out in duplicate,

a total of 16 data sets were considered. In the regression analysis, ‘coded variables’, x_i^* , were used. The latter can be obtained by a linear combination of the natural variables x_i :

$$x_i^*(x_i) = \frac{x_i - \bar{x}_i}{(x_{high} - x_{low})/2} \quad (i = 1, \dots, 3)$$

Determining the low (-1) and high (+1) levels introduced in Table S2.

PMS diameters are therefore obtained by the DoE equation:

$$\begin{aligned} Diameter = & C_0^* + C_{Cys}^* Cys^* + C_{CDI}^* CDI^* + C_{H_2O}^* H_2O^* + C_{CysCDI}^* Cys^* CDI^* + C_{CysH_2O}^* Cys^* H_2O^* \\ & + C_{CDIH_2O}^* CDI^* H_2O^* + C_{CysCDIH_2O}^* Cys^* CDI^* H_2O^* \end{aligned}$$

Using ‘coded variables’ allow to order the selected parameters in terms of their significance in the regression analysis, in our case $\{C_{Cys}^*, C_{CDI}^*, C_{H_2O}^*, C_{CysCDI}^*, C_{CysH_2O}^*, C_{CDIH_2O}^*, C_{CysCDIH_2O}^*\}$. Coded variables are orthogonal and this makes possible to evaluate each parameter independently, allowing to dismiss separately non-significant parameters without affecting the statistical confidence of the others.

The experimental input and output parameters are reported in Table S3.

Table S3 – Values of the ‘coded variables’ and relative output values

order	Input (X_i) - coded			Output (Y)
	Cys	CDI	H₂O	diameter (nm)
1	-1	-1	-1	1874
2	+1	-1	-1	3449
3	-1	+1	-1	7140
4	+1	+1	-1	8635
5	-1	-1	+1	4616
6	+1	-1	+1	4361
7	-1	+1	+1	4659
8	+1	+1	+1	6572
9	-1	-1	-1	5963
10	+1	-1	-1	3282
11	-1	+1	-1	5055
12	+1	+1	-1	2877
13	-1	-1	+1	5139
14	+1	-1	+1	4903
15	-1	+1	+1	7107
16	+1	+1	+1	1929

The statistical significance of the regression analysis was determined by evaluating the p and T parameters. p is the probability to observe a certain value within a distribution. A selected threshold of p establishes if the value analysed can be accepted or neglected. The T parameter is associated to difference between the average values provided by the experimental and reference distribution. High values of p and low absolute values of T attest the statistical significance of the data.

The quality of the adopted DoE model is assessed by the R^2 parameter, representing the percent variation of the output variable Y accounted by the applied regression analysis. A better parameter, that considers the number of regressor variables (p) with respect to the number of experimental data (n) is R_{Adj}^2 :

$$R_{Adj}^2 = 1 - (1 - R^2) \frac{p}{n - p - 1}$$

The results of the applied regression analysis using a statistical threshold $\alpha = 0.25$ for the chosen regressor variables are reported in Table S4.

Table S4 – Statistical assessment of the DoE model applied for the determination of the average diameter of NaAlg microsponges

	Regressor	Coefficients		p	T	Order of significance
	<i>Constant</i>	C_0^*	- 10925		- 2,59	
	<i>Cys</i>	C_{Cys}^*	-	> 0,25		
	<i>CDI</i>	C_{CDI}^*	-	> 0,25		
	<i>H₂O</i>	$C_{H_2O}^*$	97,5	0,011	3,05	3
	<i>Cys · CDI</i>	C_{CysCDI}^*	24,90	0,002	3,91	2
	<i>Cys · H₂O</i>	$C_{CysH_2O}^*$	- 2,669	0,002	- 4,12	1
	<i>CDI · H₂O</i>	$C_{CDIH_2O}^*$	- 1,232	0,084	- 1,90	4
	<i>Cys · CDI · H₂O</i>	$C_{CysCDIH_2O}^*$	-	> 0,25		
R^2		80,9 %				
R_{Adj}^2		74,0 %				

The values obtained for R^2 (80.9%) and R_{Adj}^2 (74.0%) make us confident on the applied DoE analysis of the experimental data.

The Pareto histograms [1] (Figure S9) report the number of data with statistical significance (standardized effects) larger than 0.25 for some of the analysed regressor variables. From this analysis, the most relevant factor is the {Cys · H₂O} interaction,

followed, with decreasing effect, by {Cys · CDI}, {H₂O} and {CDI · H₂O}. It is also possible evaluate the contribution of each factor to the output with respect to the normal distribution (Figure S9B). Coded variables showing negative values with respect to such distribution decrease the statistical significance of the output on going from the low to the high level, while positive values increase the quality of the answer. The data reported in Figure S9 show that the {Cys · H₂O} and {CDI · H₂O} regressors cause a decrease in the statistical significance of the output, while the {H₂O} and {Cys · CDI} factors increase such parameter.

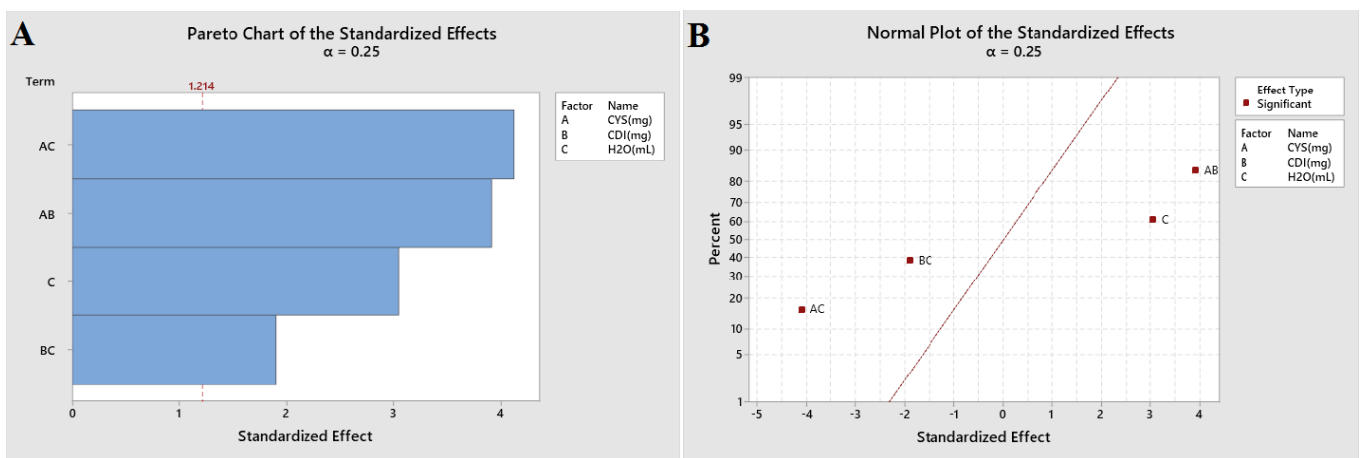


Figure S9. A) Standardized effect of the analysed coded variable (Pareto histogram); B) Deviation of the standardized effect from the normal distribution (dotted line).

The adopted statistical model (DoE), applied to the DLS experimental data sets to determine the diameter of the NaAlg PMS, provided the following equation:

$$diameter = -10925 + 97.5 H_2O + 24.90 Cys \cdot CDI - 2.669 Cys \cdot H_2O - 1.232 CDI \cdot H_2O \quad (3.7)$$

1. Montgomery D.C., *Design and Analysis of Experiments*, New York: Wiley, 2005

Table S5. Distribution analysis of DLS data for the formulations (DoE items)

DoE item	Peaks	Diam (nm)	% Intensity	Width (nm)	Z-average ¹ (d.nm)	Pdl ²
DoE 1	2	7390 2213	53.4 46.6	1092 451.2	3750	0.352
	1	4378	100	1439	3744	0.155
	2	5055 1032	96.5 3.5	1733 194.8	4160	0.172
	2	2369 6503	56.9 43.1	804.3 1442	3003	0.292

DoE 2	2	1928 7341	51.6 48.4	412.8 1112	3192	0.347
	1	4659	100	2154	3655	0.245
DoE 3	2	7571 1895	82.9 17.1	997.8 346.4	5609	0.34
	2	7673 736.5	91.1 8.9	945.9 88.0	6020	0.373
	1	2877	100	502.7	2704	0.126
DoE 4	2	7184 1754	88.7 11.3	1168 303.0	5681	0.27
	2	5989 864.1	93.4 6.6	1625 230.3	4626	0.264
	1	1929	100	248.0	1779	0.071
DoE 5	2	7960 2232	55.9 44.1	782.5 359.6	4367	0.412
	1	6082	100	1405	5341	0.209
	1	3449	100	519.5	3197	0.08
DoE 6	1	6046	100	1557	5064	0.205
	1	5095	100	1809	4286	0.191
	1	3282	100	452.0	3278	0.211
DoE 7	2	6031 1833	94.5 5.5	1645 324.5	5040	0.204
	1	5460	100	1595	4586	0.155
	1	5963	100	1490	5614	0.117
DoE 8	2	6927 1820	61.6 38.4	1273 409.7	3367	0.398
	2	6099 2198	71.2 28.8	1691 550.1	3461	0.366
	1	4903	100	1335	4444	0.084
DoE 9	1	6920	100	1237	6671	0.186
	1	8281	100	549.9	7993	0.369
	1	6572	100	1256	6516	0.073
DoE 10	1	6719	100	1557	6435	0.201
	2	8440 775.4	93.8 6.2	437.8 55.9	8244	0.256
	1	7140	100	1172	7056	0.166
DoE 11	2	8098 2024	69.6 30.4	710.6 298.7	5415	0.398
	1	7693	100	936.5	7732	0.111
	1	5139	100	934.0	5165	0.185
DoE 12	1	5079	100	1249	4677	0.149
	2	6670 3224	66.6 33.4	1486 614.3	5014	0.214
	1	4616	100	810.4	4764	0.473
DoE 13	1	6982	100	1380	6920	0.15
	2	8307 1602	91.4 8.6	528.2 138.8	7741	0.287
	1	7107	100	1176	6867	0.218
DoE 14	1	3762	100	696.9	3544	0.055
	2	7964 1467	83.3 16.7	780.6 226.9	5470	0.482
	1	1874	100	249.8	1910	0.628
	2	8319	81.6	522.4	7238	0.398

DoE 15		3243	18.4	357.4		
	1	7550	100	952.8	7351	0.186
DoE 16	2	1350	69.5	228.2	6044	0.314
	1	5560	30.5			
	1	7260	100	1345	7187	0.241

¹ $Z - average = \frac{\sum_i S_i}{\sum_i (S_i/D_i)}$ where S_i and D_i are the scattered intensity and the diameter of the particle i , respectively.

² PdI = Polydispersity index.

Section S6. CIGB814 loading to NaAlg PMS

Table S6. UV-Vis Absorption of supernatant solutions of CIGB814 (0.5 mg/ml) and NaAlg PMS (4 mg) at different times.

	<i>Incubation time (h)</i>	<i>Abs (280 nm) ± SD</i>	<i>CIGB814 concentration (supernatant) (mg/mL) ± SD</i>	<i>CIGB814 loading (%) ± SD</i>
<i>NaAlg PMS (4 mg)</i>	-	0.031 ± 0.002	-	-
<i>CIGB814 0.5 mg/mL</i>	-	0.272 ± 0.003	0.552 ± 0.006	0
<i>NaAlg2 PMS + CIGB814 (supernatant)</i>	0	0.215 ± 0.003	0.436 ± 0.006	21 ± 1
	0.5	0.222 ± 0.003	0.387 ± 0.006	30 ± 1
	1	0.225 ± 0.001	0.394 ± 0.003	28.5 ± 0.5
	2	0.215 ± 0.007	0.37 ± 0.01	32 ± 3
	3	0.208 ± 0.007	0.360 ± 0.001	34.82 ± 0.02
	4	0.216 ± 0.002	0.375 ± 0.004	31.9 ± 0.7

Table S7. UV-Vis Absorption of supernatant solutions of CIGB814 (0.5 mg/ml) and NaAlg PMS (8 mg) at different times.

	<i>Incubation time (h)</i>	<i>Abs (280 nm) $\pm SD$</i>	<i>CIGB814 Concentration (supernatant) (mg/mL) $\pm SD$</i>	<i>CIGB814 loading (%) $\pm SD$</i>
<i>Microsponges (8 mg)</i>	-	0.06 ± 0.02	-	-
<i>CIGB814 0.5 mg/mL</i>	-	0.254 ± 0.002	0.514 ± 0.003	0
<i>Microsponges+ CIGB814</i>	0	0.24 ± 0.01	0.48 ± 0.02	6 ± 4
	0.5	0.144 ± 0.004	0.160 ± 0.007	69 ± 1
	1	0.16 ± 0.02	0.19 ± 0.04	61 ± 7
	2	0.159 ± 0.003	0.190 ± 0.006	63 ± 1
	3	0.15 ± 0.02	0.17 ± 0.05	66 ± 9
	4	0.16 ± 0.02	0.18 ± 0.04	64 ± 7

Table S8. Fluorescence emission intensities and fractional loading (%) of supernatant solutions of CIGB814 (0.5 mg/ml) and NaAlg PMS (4 mg) at different times.

	<i>Incubation time (h)</i>	<i>Fluorescence intensity ($\lambda_{em}=307$ nm) $\pm SD$</i>	<i>CIGB814 loading (%) $\pm SD$</i>
<i>NaAlg PMS (4 mg)</i>	-	41769 ± 1067	-
<i>CIGB814 0.5 mg/mL</i>	-	$2.49 \pm 0.01 \text{ E}+06$	0
<i>NaAlg PMS + CIGB814</i>	0	$2.22 \pm 0.11 \text{ E}+06$	12 ± 5
	0.5	$1.73 \pm 0.02 \text{ E}+06$	32.1 ± 0.9
	1	$1.64 \pm 0.04 \text{ E}+06$	36 ± 2
	2	$1.58 \pm 0.01 \text{ E}+06$	38.3 ± 0.4
	3	$1.57 \pm 0.07 \text{ E}+06$	38 ± 3
	4	$1.60 \pm 0.01 \text{ E}+06$	37 ± 0.4

Table S9. Fluorescence emission intensities and fractional loading (%) of supernatant solutions of CIGB814 (0.5 mg/ml) and NaAlg PMS (8 mg) at different times.

	<i>Incubation time (h)</i>	<i>Fluorescence intensity (307 nm) ± SD</i>	<i>CIGB814 loading (%) ± SD</i>
<i>NaAlg PMS (8 mg)</i>	-	16789 ± 1090	-
<i>CIGB814 (0.5 mg/ml)</i>	-	2.49 ± 0.01 E+06	0
<i>NaAlg PMS + CIGB814</i>	0	1.57 ± 0.26 E+06	37 ± 10
	0.5	7.44 ± 0.25 E+05	70 ± 1
	1	7.02 ± 0.35 E+05	72 ± 1
	2	6.08 ± 0.18 E+05	75.5 ± 0.7
	3	5.72 ± 0.43 E+05	77 ± 2
	4	5.52 ± 0.14 E+05	77.8 ± 0.6

Section S7. CIGB814 release from NaAlg2 PMS in PBS pH=7.4

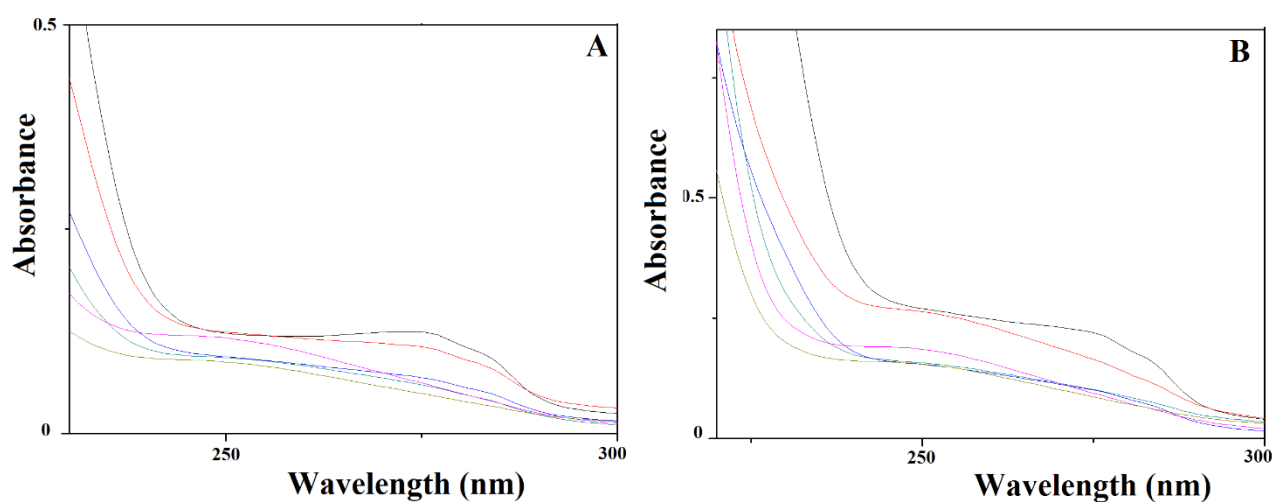


Figure S10. UV-Vis absorption spectra of supernatant solutions of CIGB814/NaAlg PMS for increasing incubation times in PBS (pH=7.4). CIGB814: 0.5 mg/mL; NaAlg PMS: A) 4 mg/mL; B) 8 mg/mL. Black: 15 min; Red: 1 h; Blue: 2 h; Emerald: 24 h; Purple: 92 h; Olive: 116 h.

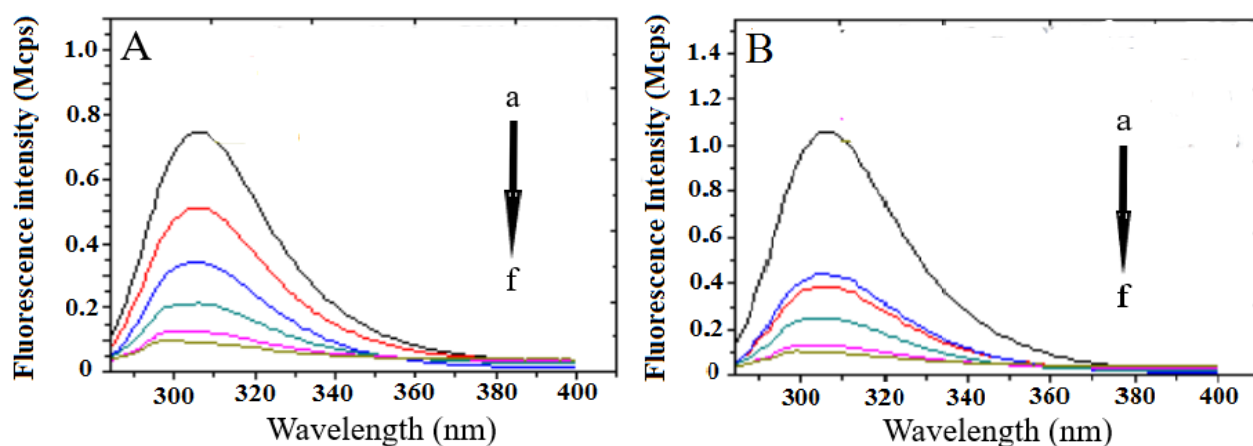


Figure S11. Fluorescence emission spectra (Mcps=106 count per second) of CIGB814/NaAlg PMS supernatant solutions at increasing incubation times in PBS (pH=7.4). A) CIGB814: 0.19 mg/mL; NaAlg PMS: 4 mg/mL; B) CIGB814: 0.37 mg/mL; NaAlg PMS: 8 mg/mL (C). a) 15 min; b) 1 h; c) 2 h; d) 24 h; e) 92 h; f) 116 h.

Table S10. Fluorescence emission data and fractional release (%) of supernatant solutions of NaAlg PMS (4 mg)/CIGB814 (0.5 mg/ml) in PBS pH 7.4 at different incubation times.

	<i>Incubation time (h)</i>	<i>Fluorescence Intensity (cps) ($\lambda_{em}=307\text{ nm}$) $\pm SD$</i>	<i>CIGB814 release (%) $\pm SD$</i>
<i>NaAlg PMS (4 mg)</i>	0.25	25419 \pm 1091	-
	1	38244 \pm 1089	-
	2	29978 \pm 1090	-
	24	30285 \pm 1007	-
	92	46595 \pm 1091	-
	116	51367 \pm 1080	-
<i>Loaded CIGB814</i>	-	1.39 \pm 0.41 E+06	100
<i>NaAlg + CIGB814 (PBS, pH 7.4)</i>	0.15	7.48 \pm 0.71 E+05	52 \pm 5
	1	5.09 \pm 0.13 E+05	59.8 \pm 0.9
	2	3.40 \pm 0.13 E+05	71.0 \pm 0.9
	24	2.13 \pm 0.09 E+05	73.0 \pm 0.9
	92	1.24 \pm 0.05 E+05	73.0 \pm 0.9
	116	9.29 \pm 0.44 E+04	73.2 \pm 0.9

Table S11. Fluorescence emission data and fractional release (%) of supernatant solutions of NaAlg PMS (8 mg)/CIGB814 (0.5 mg/ml) in PBS pH 7.4 at different incubation times.

	<i>Incubation time (h)</i>	<i>Fluorescence Intensity (cps) ($\lambda_{em}=307\text{ nm}$) $\pm SD \pm SD$</i>	<i>CIGB814 release (%) $\pm SD$</i>
<i>NaAlg PMS (8 mg)</i>	0.25	37854 \pm 1050	-
	1	65023 \pm 1009	-
	2	27522 \pm 1032	-
	24	33132 \pm 1006	-
	92	39315 \pm 1090	-
	116	51220 \pm 1023	-
<i>CIGB814 loaded</i>	-	1.98 \pm 0.41 E+06	100
<i>NaAlg PMS + CIGB814 + PBS (pH 7.4)</i>	0.25	1.06 \pm 0.09 E+06	51 \pm 5
	1	6.62 \pm 0.66 E+05	56 \pm 3
	2	4.37 \pm 0.28 E+05	66 \pm 3
	24	2.50 \pm 0.08 E+05	67 \pm 3
	92	1.29 \pm 0.09 E+05	67 \pm 3
	116	9.79 \pm 0.72 E+04	67 \pm 3