Electronic Supplementary Material (ESI) for Nanoscale Advances.

Unravelling the role of individual components in pBAE/polynucleotide polyplexes: on the road to rational formulations

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No conflict of interest to be declared

Table S1. *Polyplexes sizes in mean and standard deviation, and PDI, from AFM measurements.*

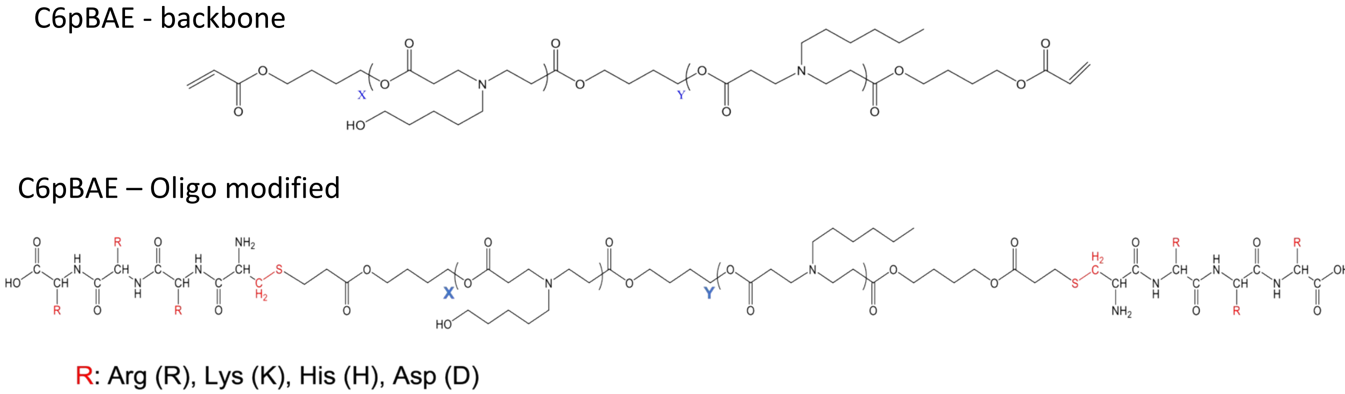
	Equivalent Diameter from AFM* **(nm)**	**Stdev**	**Calculated PDI**
KH	140.3	84.19	0.3599
KHD	91.38	52.11	0.3251
RH	115.6	108.9	0.8874
RHD	153.2	157.5	1.057
RK	116.4	94.27	0.6560
RKD	197.8	109.58	0.3068

Although not used for this purpose, AFM analysis enabled to measure polyplexes sizes by measuring the height of each polyplex after being deposited onto mica. To remark that it was assumed conservation of the volume before and after mica deposition. Equivalent sizes correspond to the mean value of, at least, 20 polyplexes for each nanoparticle type, as well as standard deviation. From them, an apparent polydispersity index (PDI) was calculated. In the majority of the polyplexes, size trend is equal comparing DLS and AFM measurements, as plotted in the next graph.

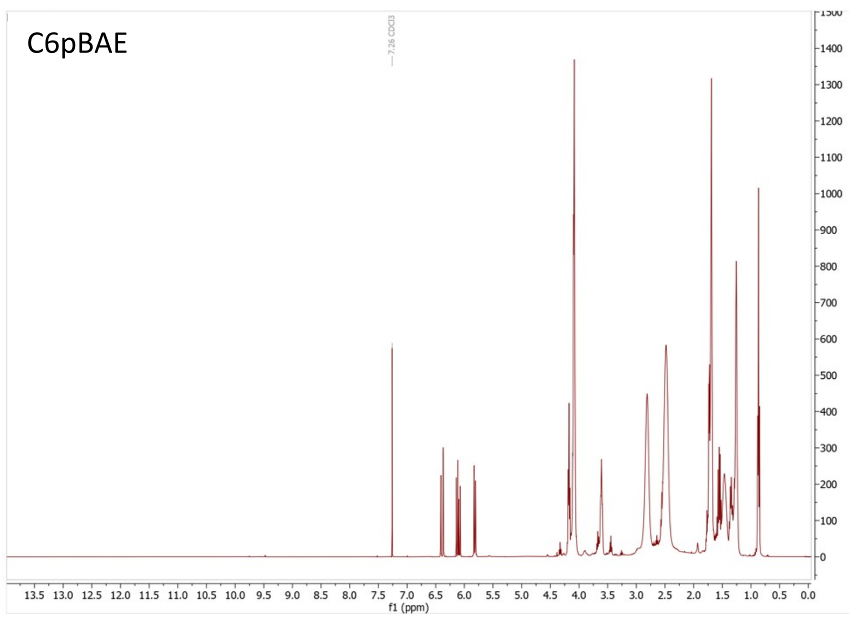
Table S2. *Nanoparticles constructions stability at different temperatures. Nanoparticles constructs incubated at different timings and temperatures (25ºC and 37ºC) with PBS. Raw data without normalization.*

			Time (h)							
			0	2	4	6	8	10	12	24	48
PBS	25	K*H/pGFP*	27685 ± 449	26619 ± 915	26610 ± 1075	26775 ± 1303	26469 ± 2184	25399 ± 3960	23542 ± 4722	22143 ± 4566	21987 ± 3755
		KH*/pGFP*	24885 ± 1600	21098 ± 1919	15067 ± 2821	10781 ± 2910	7993 ± 2445	5944 ± 1660	5383 ± 949	4976 ± 568	4454 ± 454
		K*H*/pGFP	639 ± 43	564 ± 60	570 ± 85	620 ± 116	423 ± 45	523 ± 90	501 ± 28	525 ± 29	510 ± 44
		K*HD*/pGFP	729 ± 30	753 ± 12	812 ± 71	740 ± 46	814 ± 60	708 ± 8	662 ± 54	511 ± 30	404 ± 1
		KH*D*/pGFP	514 ± 50	480 ± 28	545 ± 45	549 ± 42	421 ± 57	395 ± 40	454 ± 50	420 ± 73	347 ± 56
		KHD*/pGFP*	4709 ± 66	3939 ± 97	3551 ± 171	3185 ± 123	2954 ± 90	2723 ± 128	2484 ± 104	2252 ± 178	1745 ± 282
		R*H/pGFP*	8891 ± 487	7914 ± 310	7457 ± 197	7270 ± 273	7079 ± 536	7073 ± 249	7005 ± 150	6977 ± 235	6823 ± 177
		RH*/pGFP*	8430 ± 378	8244 ± 352	8131 ± 422	8139 ± 87	7915 ± 375	7982 ± 274	7545 ± 611	7212 ± 199	6988 ± 324
		R*H*/pGFP	839 ± 117	706 ± 29	702 ± 79	772 ± 111	712 ± 128	819 ± 74	720 ± 102	721 ± 56	713 ± 88
		R*HD*/pGFP	4198 ± 132	3528 ± 219	3399 ± 239	3383 ± 200	3196 ± 232	3003 ± 77	2840 ± 298	2712 ± 206	2599 ± 232
		RH*D*/pGFP	3073 ± 198	2448 ± 109	2292 ± 154	2252 ± 72	2111 ± 215	2076 ± 159	2014 ± 43	1965 ± 180	1965 ± 77
		RHD*/pGFP*	10626 ± 407	6241 ± 349	6131 ± 75	6011 ± 160	5994 ± 310	5579 ± 299	5474 ± 305	4859 ± 424	3765 ± 253
		R*K/pGFP*	16740 ± 686	16056 ± 526	15022 ± 273	14420 ± 462	14020 ± 202	13713 ± 738	13032 ± 983	13009 ± 951	10860 ± 221
		RK*/pGFP*	11160 ± 247	10957 ± 401	10700 ± 499	10535 ± 691	10121 ± 76	9870 ± 461	8963 ± 576	9161 ± 609	8750 ± 511
		R*K*/pGFP	2965 ± 43	3096 ± 83	3250 ± 55	3321 ± 47	3533 ± 103	3681 ± 38	3634 ± 176	2963 ± 76	2334 ± 63
		R*KD*/pGFP	506 ± 139	435 ± 139	389 ± 130	424 ± 130	391 ± 92	342 ± 86	351 ± 91	295 ± 31	279 ± 34
		RK*D*/pGFP	578 ± 13	493 ± 31	488 ± 34	434 ± 4	421 ± 19	425 ± 32	389 ± 37	284 ± 113	351 ± 104
		RKD*/pGFP*	8685 ± 230	8721 ± 253	8225 ± 314	7163 ± 513	5589 ± 517	3915 ± 426	2716 ± 291	987 ± 87	617 ± 129
	37	K*H/pGFP*	21407 ± 337	5933 ± 368	4376 ± 400	3860 ± 106	3576 ± 50	3430 ± 260	3107 ± 78	3115 ± 198	3132 ± 599
		KH*/pGFP*	15565 ± 337	3928 ± 529	3290 ± 188	3118 ± 85	3071 ± 138	2954 ± 183	2900 ± 208	2774 ± 181	2898 ± 133
		K*H*/pGFP	428 ± 19	167 ± 123	169 ± 33	105 ± 110	126 ± 88	145 ± 140	178 ± 36	188 ± 74	105 ± 50
		K*HD*/pGFP	4198 ± 132	3528 ± 219	3399 ± 239	3383 ± 200	3196 ± 232	3003 ± 77	2840 ± 298	2712 ± 206	1730 ± 50
		KH*D*/pGFP	3073 ± 198	2448 ± 109	2292 ± 154	2252 ± 72	2111 ± 215	2076 ± 159	2014 ± 43	1965 ± 180	1196 ± 18
		KHD*/pGFP*	4709 ± 66	3939 ± 97	3551 ± 171	3185 ± 123	2954 ± 90	2723 ± 128	2484 ± 104	2252 ± 178	1213 ± 512
		R*H/pGFP*	6626 ± 446	1592 ± 92	1248 ± 136	1303 ± 23	1304 ± 40	1300 ± 61	1255 ± 60	1140 ± 166	1040 ± 52
		RH*/pGFP*	6627 ± 309	2914 ± 342	1566 ± 347	1093 ± 188	1234 ± 117	961 ± 71	1032 ± 15	1059 ± 157	997 ± 91
		R*H*/pGFP	257 ± 93	180 ± 94	58 ± 52	136 ± 28	165 ± 10	92 ± 65	112 ± 16	103 ± 84	357 ± 115
		R*HD*/pGFP	5341 ± 625	3607 ± 88	3321 ± 28	3091 ± 40	3058 ± 269	2744 ± 258	2489 ± 433	1361 ± 64	1321 ± 51
		RH*D*/pGFP	2969 ± 813	2017 ± 725	1951 ± 802	1911 ± 754	1810 ± 790	1850 ± 843	1730 ± 838	1511 ± 993	1147 ± 213
		RHD*/pGFP*	10579 ± 1905	6788 ± 31	6597 ± 334	6287 ± 167	5924 ± 16	5601 ± 514	5077 ± 910	2450 ± 434	2473 ± 1002
		R*K/pGFP*	19356 ± 979	13771 ± 134	13378 ± 206	11125 ± 303	9854 ± 197	9725 ± 21	9661 ± 109	9467 ± 21	7700 ± 50
		RK*/pGFP*	9868 ± 42	8464 ± 321	8408 ± 317	7301 ± 83	7090 ± 206	7300 ± 3	7585 ± 19	7853 ± 31	7435 ± 116
		R*K*/pGFP	2876 ± 162	2030 ± 136	2028 ± 226	1683 ± 31	1547 ± 105	1557 ± 35	1495 ± 64	1459 ± 66	1153 ± 56
		R*KD*/pGFP	822 ± 13	585 ± 23	541 ± 32	421 ± 16	394 ± 13	398 ± 19	373 ± 12	349 ± 15	366 ± 11
		RK*D*/pGFP	805 ± 13	593 ± 32	542 ± 39	454 ± 22	436 ± 16	420 ± 20	395 ± 32	357 ± 23	357 ± 16
		RKD*/pGFP*	5789 ± 140	2525 ± 302	1941 ± 181	4047 ± 220	1385 ± 61	1237 ± 23	1159 ± 26	1208 ± 55	2612 ± 254

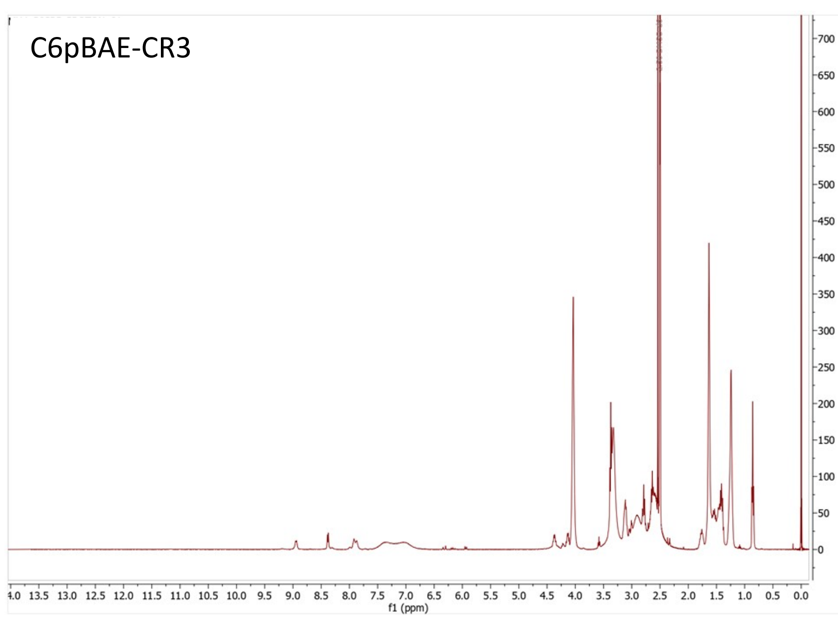
A


C6pBAE - backbone
C6pBAE – Oligo modified
R: Arg (R), Lys (K), His (H), Asp (D)

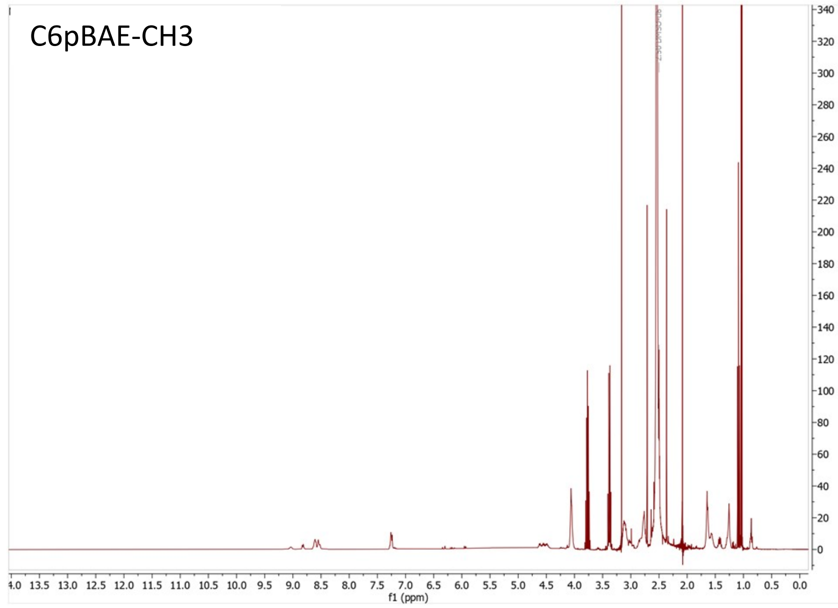
B


C6pBAE
7.26 CDCl3
f1 (ppm)

C


C6pBAE-CR3
f1 (ppm)

D



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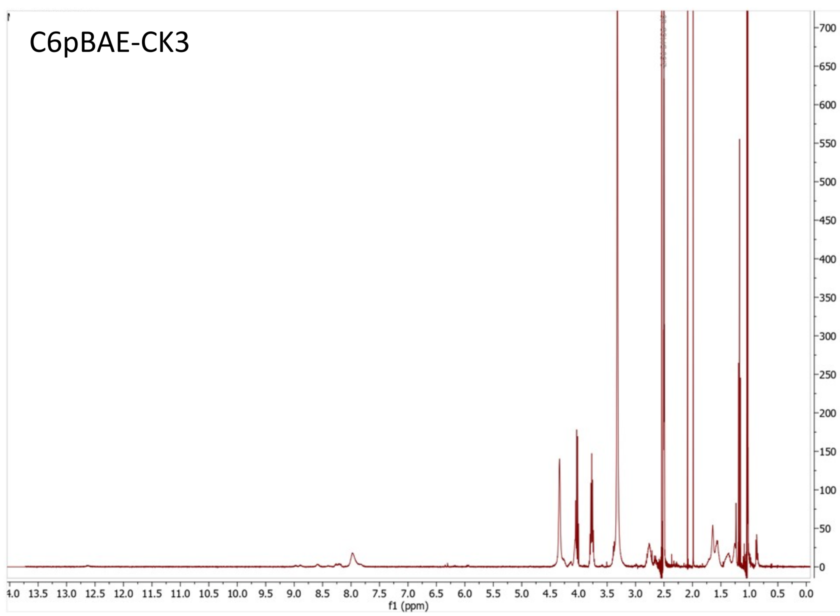

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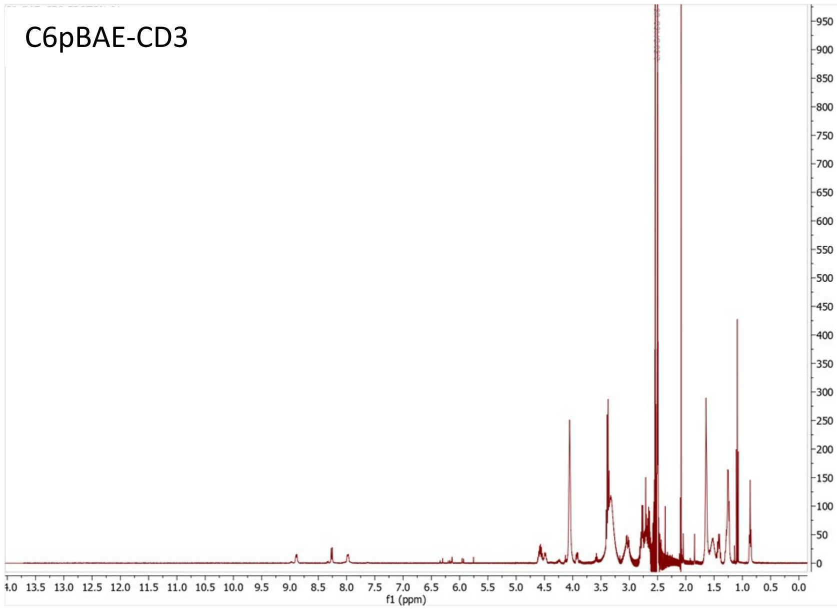

G

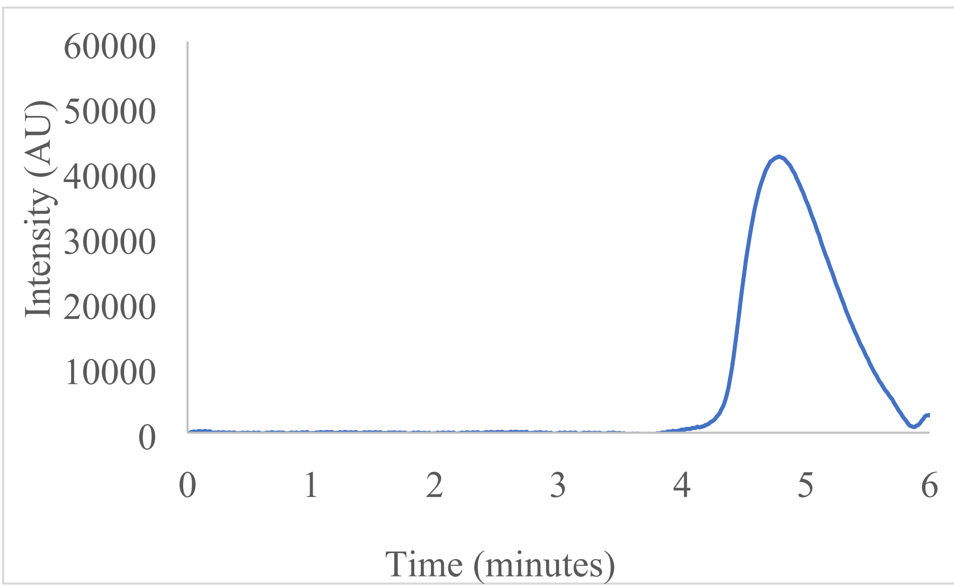

Figure S1. *Polymers characterization: A - Chemical structure of the C6-pBAE backbone and the OM-pBAE with the different peptide combination; B – F: 1H-NMR characterization of the four polymers used: B – C6; C – C6CR3; D – C6CH3; E – C6CK3; F – C6CD3; and G- GPC analysis of the backbone polymer.*

C6 polymers were characterized by NMR (structure). Additionally, first, prior to the addition of the oligopeptides. Since all the oligopeptide end-modified pBAE come from the same C6 template, we have conducted the GPC (HPSEC) characterisation of this chain before the oligopeptides attachment.

Molecular weight determination was conducted on a Hewlett-Packard 1050 Series HPLC system equipped with twoGPC Ultrastyragel columns, 10^3 and 10^4 Å (5 µm mixed, 300 mm x 19 mm, Waters Millipore Corporation, Milford, MA, USA) and THF as mobile phase. The molecular weight was calculated by comparison with the retention times of polystyrene standards.

Polymerization was confirmed by HPLC-SEC and the resulting C6 polymer had an average molecular weight (Mw) of approximately 2,500 g/mol (relative to polystyrene standards) and a polydispersity (M_w/M_n) of 1.81, showing a rather broad statistical distribution of polymer chain lengths.

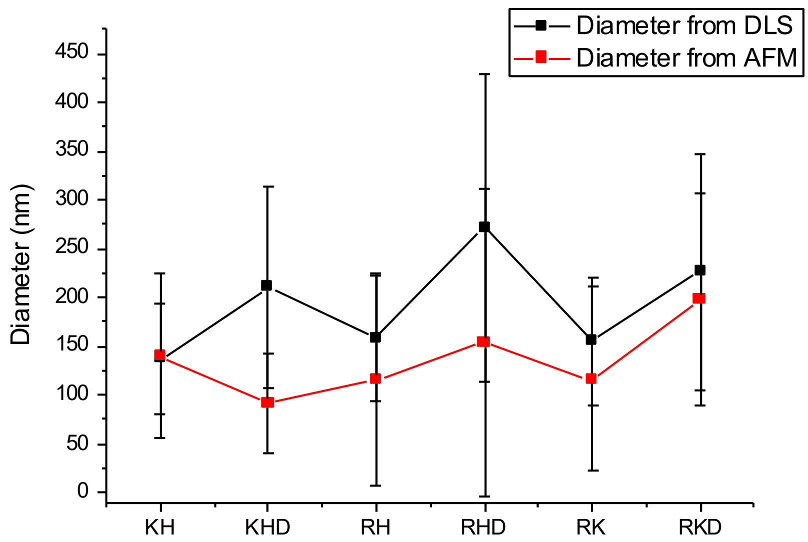

***Figure S2. AFM diameter values of the nanoparticles compared to the obtained with the DLS**. (AFM tip correction has been performed).*

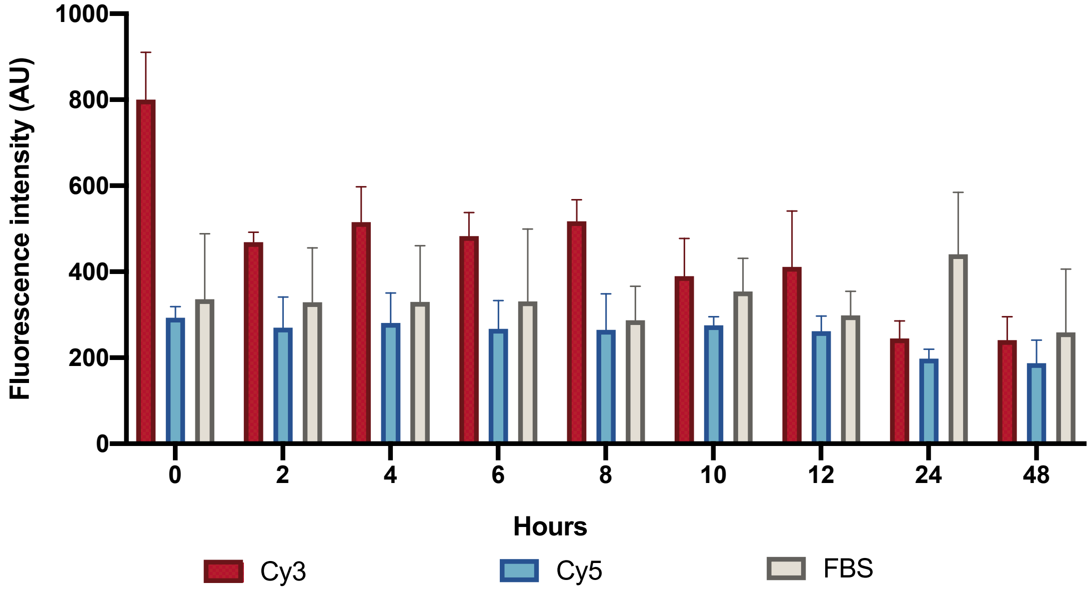

Figure S3. FRET controls. *Fluorescent intensity of the control fluorophores obtained with FRET experiments. Raw data without normalization.*

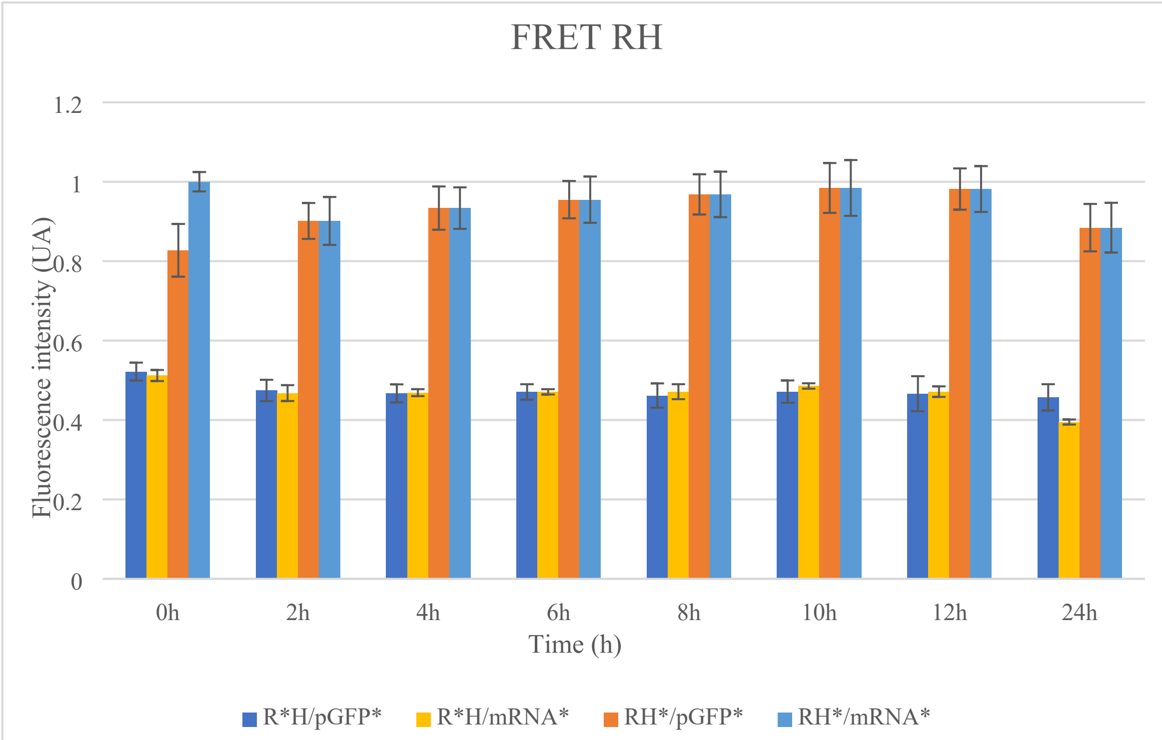

Figure S4. FRET experiment RH nanoparticles with and different genetic materials (pGFP and mRNA).

Figure S5. Biocompatibility results. *A – Hemolysis of a representative cationic (KH) and anionically-coated (KHD) NP and B – Cell biocompatibility by MTT of all the combinations.*

A

Sample	Hemolysis (%)
Water (C+)	100
PBS (C-)	0
KH	4,15
KHD	1,62

B

	Cell Viability (%)	± SD (%)
C-	100	0,17
RK	117	0,06
RKD	107	0,03
KH	98	0,06
KHD	107	0,08
RH	114	0,06
RHD	95	0,00

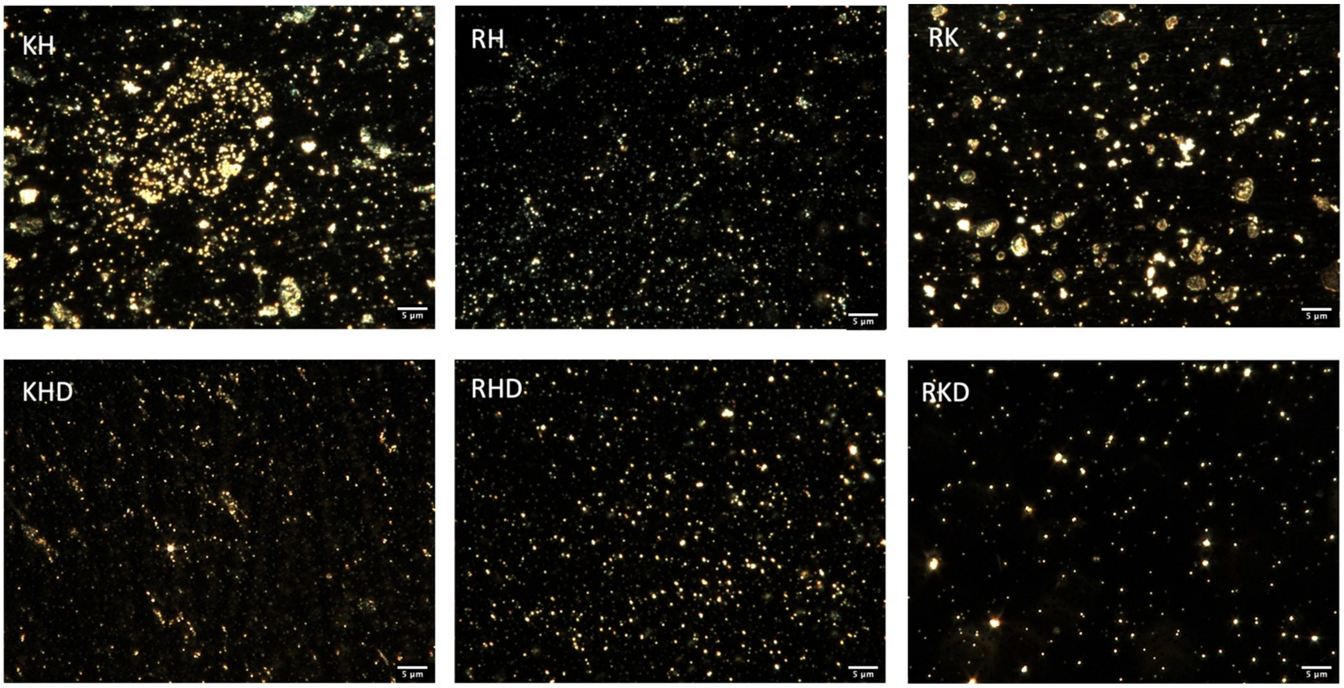

***Figure S6**. **Polyplexes optical images**. Optical images of the different nanoparticles' samples. Images were obtained with a 10X objective. Scale bars = 5µm.*

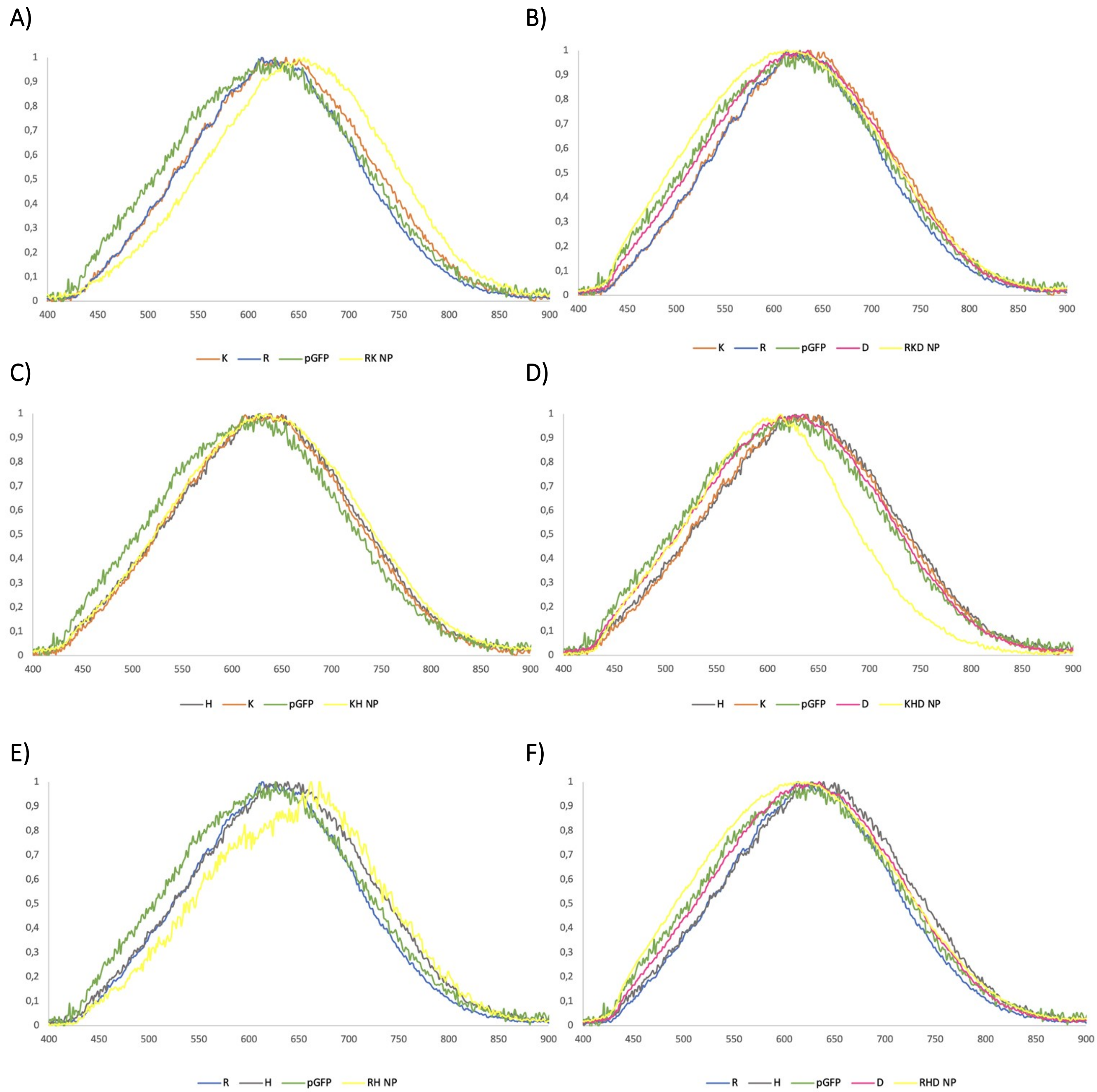

Figure S7. Cytoviva's hyperspectral profile of the different samples.. *A)-B) illustrates KH and KHD nanoparticles components respectively, C)-D) RH and RHD and E)-F) RK and RKD.*