Supporting Information

Protein−Sugar-Glass Nanoparticle Platform for the Development of Sustained-Release Protein Depots by Overcoming Protein Delivery Challenges

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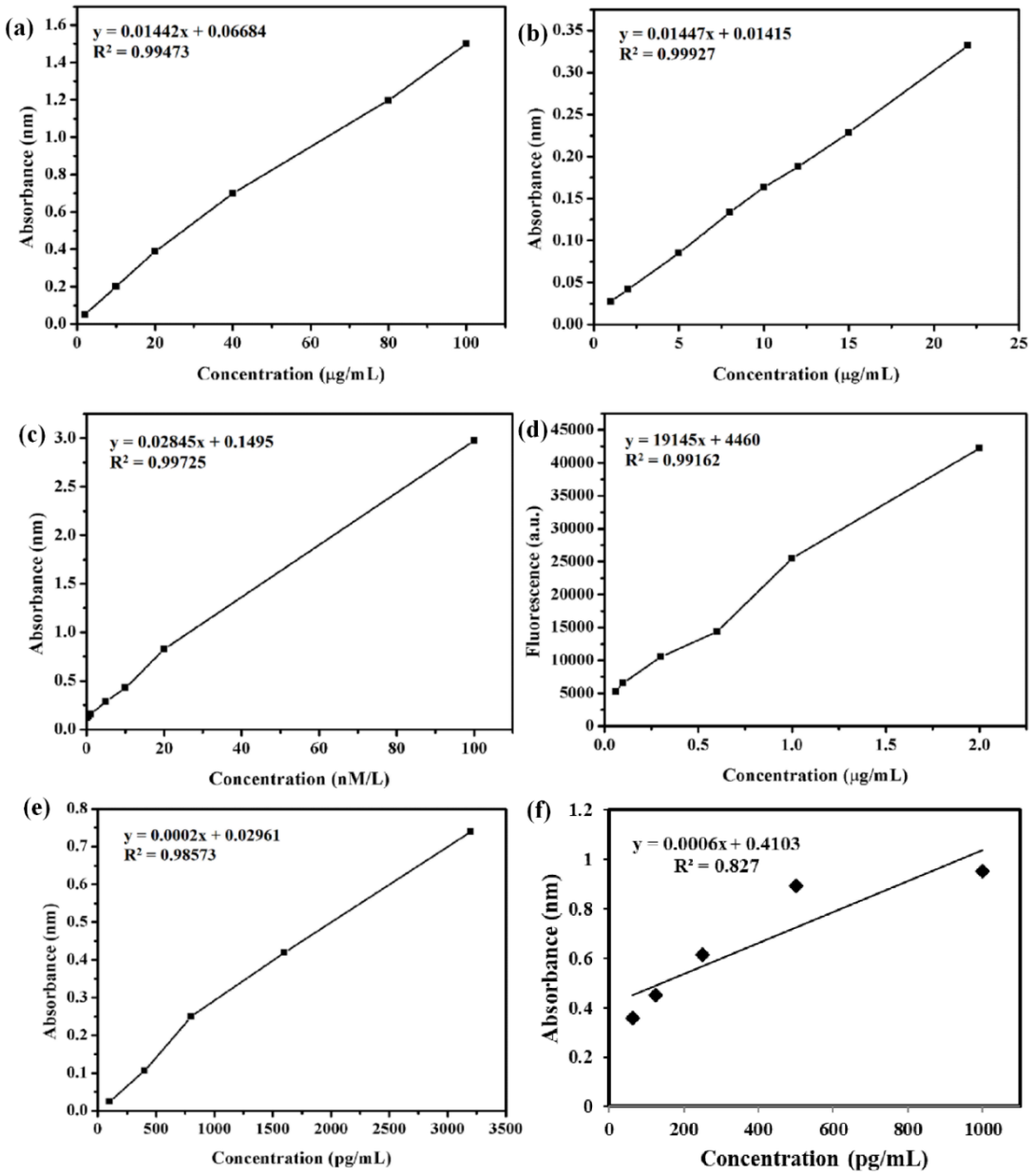

Figure S1: (a) Standard plot for total BSA quantification in the range of 2 µg/mL to 100 µg/mL by micro BSA Assay kit (Pierce). (b) Standard plot for total HRP quantification within the range between 2 µg/mL to 25µg/mL by micro BSA Assay kit. (c) Standard plot for active HRP quantification ranging between 1 nM/L to 100nM/L by the enzymatic activity assay of HRP using OPD substrate. (d) Standard plot for total FGF-2/EGF/HRP quantification (ELISA active+inactive fraction) within the range between 0.02 µg/mL to 2 µg/mL using NanoOrange. (e) Standard plot of active FGF-2 quantification using sandwich ELISA, (f) Standard plot of active EGF quantification using sandwich ELISA.

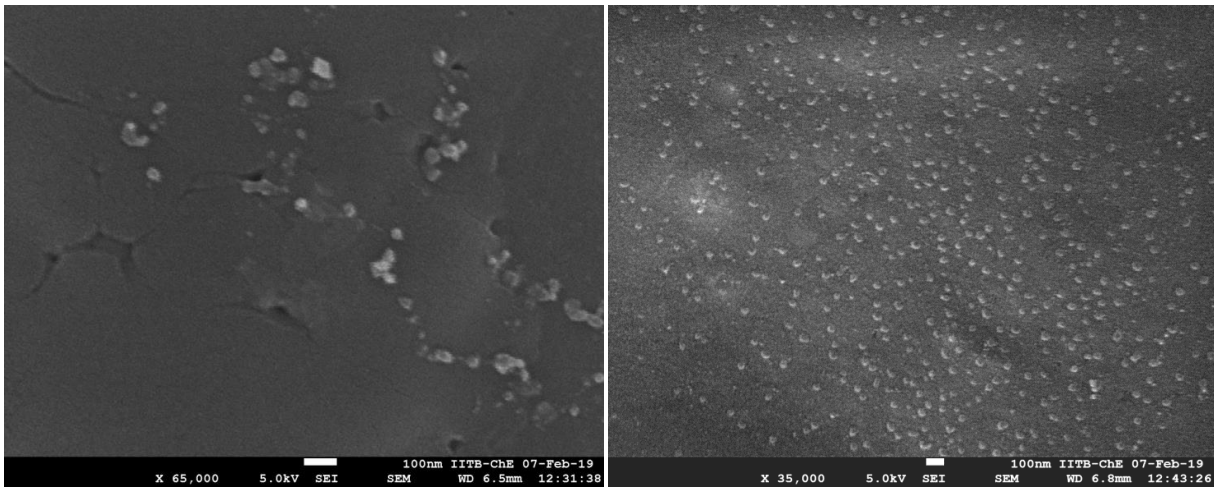

Figure S2: Cryo-SEM images of BSA-SGnP with different amount of Trehalose:BSA. (a) Trehalose:BSA is 20:1 (w/w) and, (b) Trehalose:BSA is 200:1(w/w)

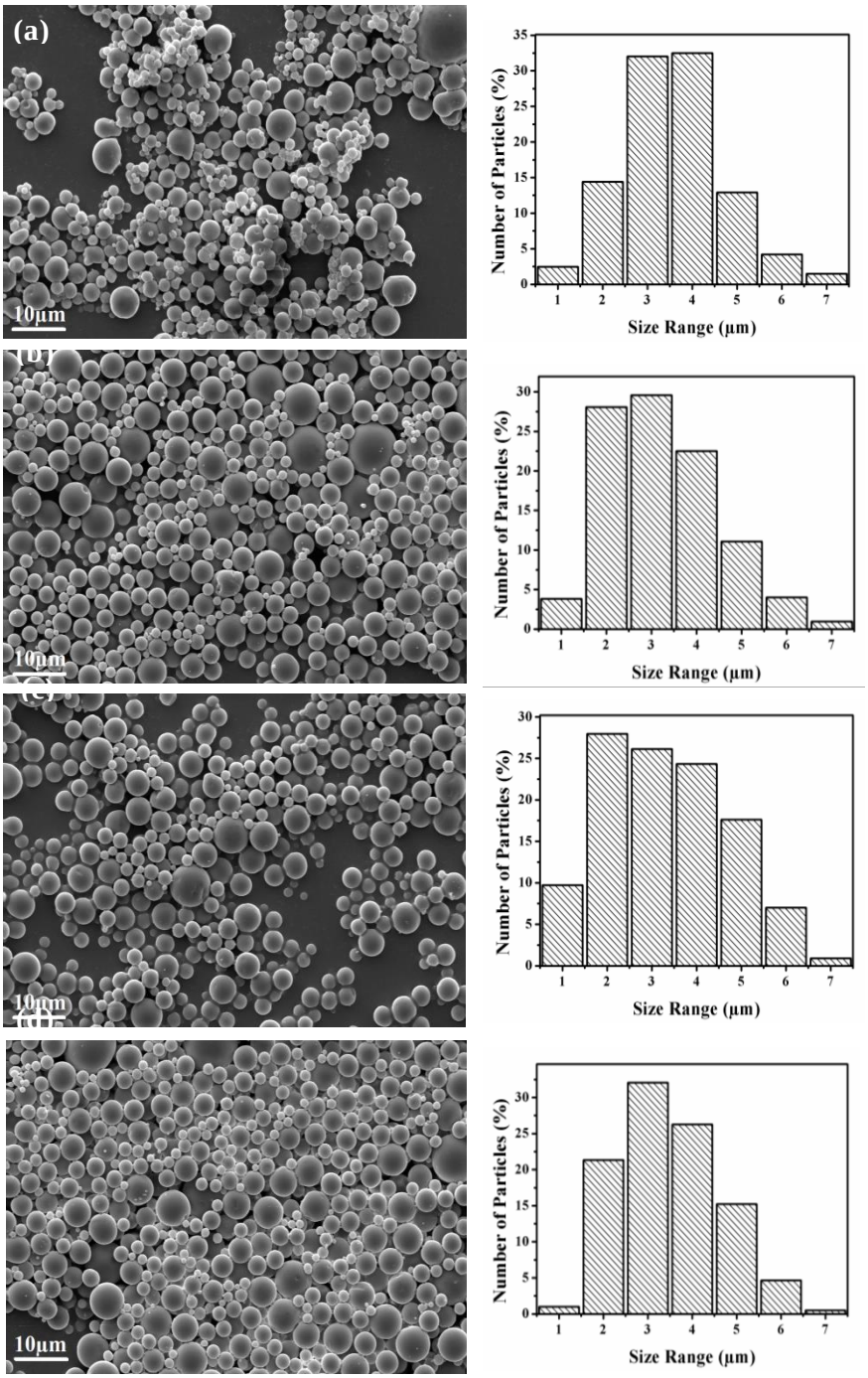

Figure S3: (a) SEM micrograph of w-o-w microparticles and their size distribution (b) SEM micrograph of sg-o-w microparticles and corresponding size distribution. (c) SEM micrograph of w-o-o microparticles and corresponding size distribution. (d) SEM micrograph of sg-o-o microparticles and corresponding size distribution. All samples shown in this figures are BSA loaded microparticles with target loading of 1 %.

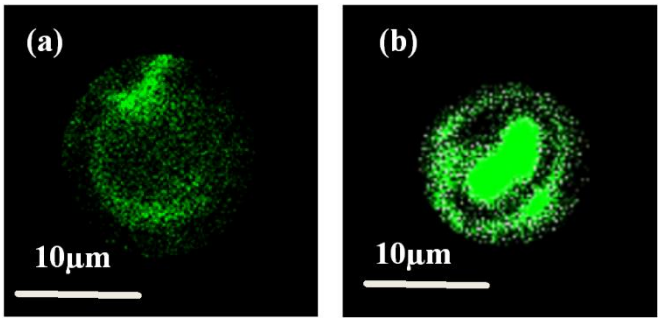

Figure S4: (a) FITC-BSA distribution within the particles prepared by conventional emulsion technique (w-o-o) (b) FITC-BSA distribution in particles prepared by our SGnP-mediated emulsion technique (sg-o-o).

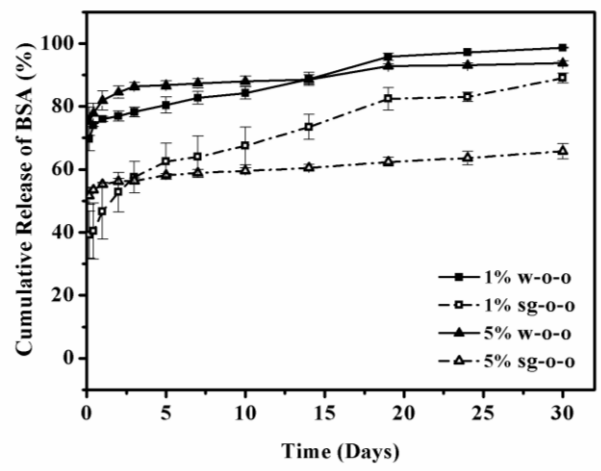

Figure S5: Cumulative release of BSA from PLGA microparticles prepared by w-o-o method with different theoretical target loading of protein 1% (w/w) and 5% (w/w).

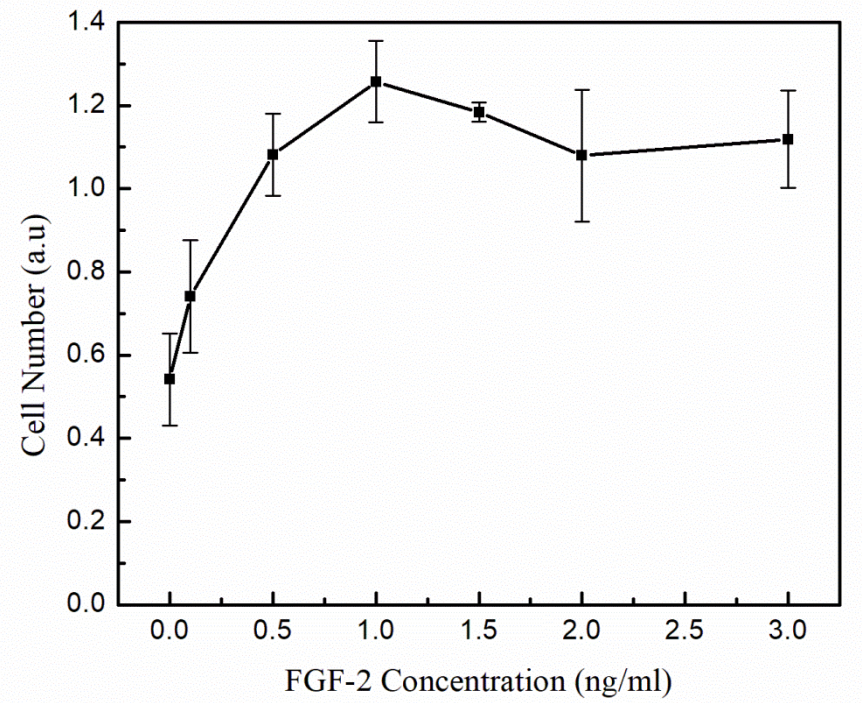

Figure S6: FGF-2 GF dose dependent proliferation of HGF cultured for 48 h in presence of FGF-2

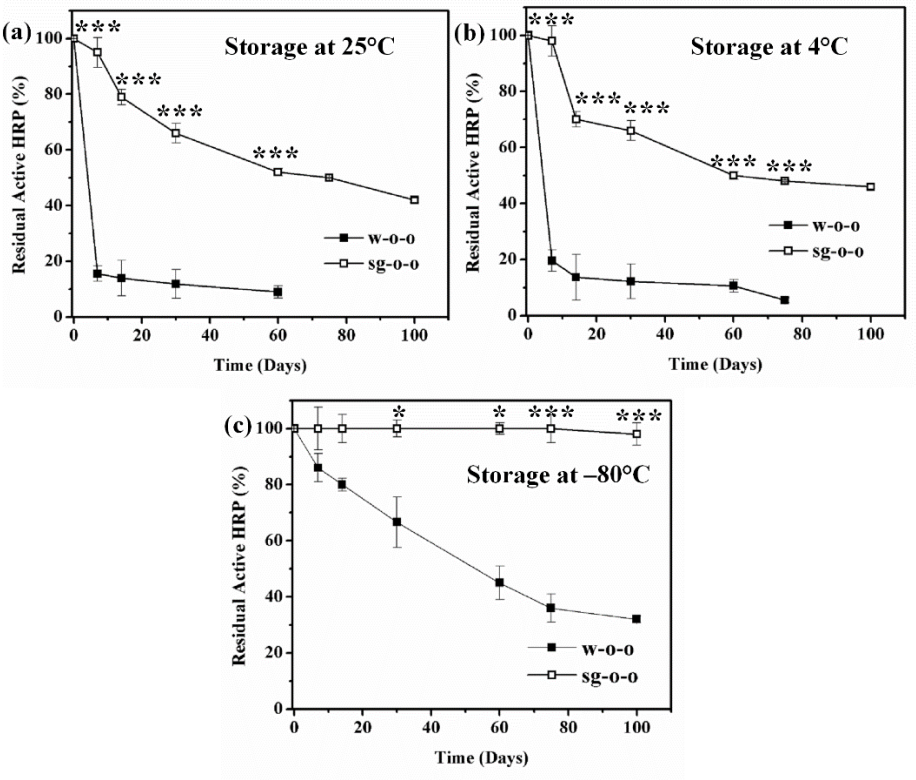

Figure S7: Three months storage stability of HRP in the different microparticles (w-o-o and sg-o-o) stored at three different temperature. Residual active HRP in the different microparticles, (a) stored at 25ºC. (b) stored at 4ºC. (c) stored at –80ºC. Residual HRP activity at each time point is compared for significance between conventional and sugar glass mediated microparticles systems. Results indicates that SGnP incorporation data is extremely significant (***P<0.001) at majority of the study points. Here, ***P<0.001, **P<0.01, *P<0.05.

(a)

Emulsion method	Theoretical loading (%)	BSA released on 14th day (%)	Amount of BSA remaining (µg)	Recovered BSA (µg)
w-o-w	1	71 ± 1.6	9.7	8.13
	5	58 ± 6	33	30.44
sg-o-w	1	31 ± 1.4	42	33.68
	5	22 ± 2	137	126

(b)

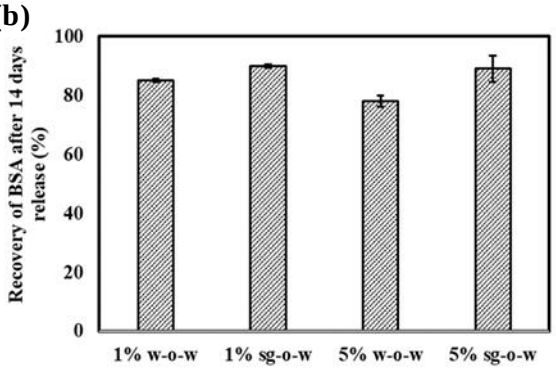

Figure S8: (a) Representing the data related to released (%) and remaining amount of BSA (microgram) after released from the two different particles, w-o-w and sg-o-w. Amount of recovered BSA from the particles kept for 14 days of release (microgram). (b) Percentage of recovery (percent) of BSA from the different particles (w-o-w and sg-o-w)

Extraction of unreleased protein after *in vitro* release study: We have studied 14 days release of BSA from w-o-w and sg-o-w particles with theoretical BSA loading capacity of 1% and 5%. After the final released for 14 days, the microparticles were retrieved and subsequently digested to release the remaining BSA inside. We have quantified the BSA and the percentage of protein recovery.

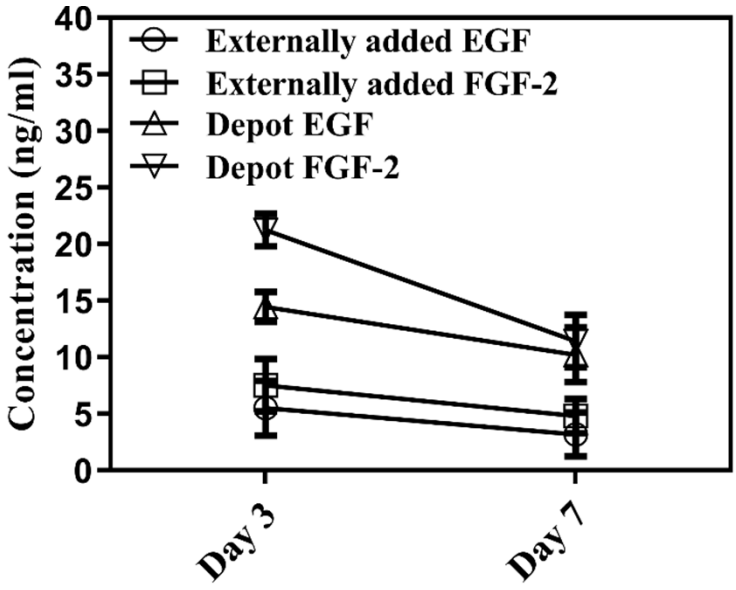

Figure S9: FGF-2 and EGF concentration in the stem cell culture medium at Day 3 and Day 7

Table S1. Summary of different ingredients used in the preparation of different protein loaded SGnPs

Protein	*[H_2O]/[AOT] mole ratio*	*Amount of protein (mg)*	*Protein: Trehalose mass ratio*
BSA	10: 1	9.2	1: 20
	10: 1	3.68	1: 50
	10: 1	2.3	1: 100
	10:1	1.15	1: 200
HRP	10: 1	0.58	1: 200
FGF-2	10: 1	0.05	1: 200
EGF	10: 1	0.05	1: 200
FITC-BSA	10: 1	0.58	1: 200
Alexa Fluor 647-BSA	10: 1	0.58	1:200

Table S2: Formulation and constituent details used for the preparation of microparticles by w-o-w method

Protein	*Theoretical loading (%)*	*Amount of protein (µg)*	*Volume of aqueous protein solution (µL)*	*Weight of PLGA (mg)*
BSA	0.008	4	200	50
BSA, FGF-2, EGF & EGF + FGF-2	0.01	5	200	50
BSA	0.05	25	200	50
HRP	0.1	50	200	50
BSA	1	500	200	50
BSA	5	2500	200	50
BSA	8	4000	200	50
BSA	15	7500	200	50

Table S3: Formulation and constituent details used for the preparation of different w-o-o microparticles

Protein	*Theoretical loading (%)*	*Amount of protein (µg)*	*Volume of aqueous protein solution (µL)*	*Weight of PLGA (mg)*
BSA	1	500	200	50
BSA	5	2500	200	50
HRP	0.1	50	200	50

Table S4: Formulation and constituent details used for the preparation of different sg-o-w microparticles

Protein	*Theoretical loading (%)*	*Amount of protein (µg)*	*Protein: Trehalose mass ratio used*	*Weight of SGnPs containing required amount of protein (mg)*	*Weight of PLGA (mg)*	*Percentage loading of SGnPs into PLGA (%)*
BSA	0.008	4	1:200	0.8	50	1.6
BSA, FGF-2, EGF & EGF + FGF-2	0.01	5	1:200	1	50	2
BSA	0.05	25	1:200	5	50	10
HRP	0.1	50	1:200	10	50	20
BSA	1	500	1:100	40	50	80
BSA	5	2500	1:20	15	50	30
BSA	8	4000	1:20	24	50	48
BSA	15	7500	1:20	45	50	90

Table S5: Formulation and constituent details used for the preparation of different sg-o-o microparticles

Protein	*Theoretical loading (%)*	*Amount of protein (µg)*	*Protein: Trehalose mass ratio used*	*Weight of SGnPs containing required amount of protein (mg)*	*Weight of PLGA (mg)*	*Percentage loading of SGnPs into PLGA (%)*
BSA	1	500	1:20	3	50	6
BSA	5	2500	1:20	15	50	30
HRP	0.1	50	1:200	10	50	20

Table S6. Summary of the encapsulation efficiency, loading capacity (target and actual), and burst release of high BSA loading formulation (1% and 5 %) from w-o-o and sg-o-o microparticles

Protein	*Emulsion method*	*Theoretical Loading (%)*	*Encapsulation Efficiency (%)*	*Loading Capacity (%)*	*Release at 24th hour (%)*
BSA	***w-o-o***	***1***	72 ± 0.23	0.7 ± 1.44	76 ± 0.81
		5	48 ± 0.08	0.9 ± 0.36	47 ± 9.02
	sg-o-o	***1***	93 ± 0.12	0.9 ± 2.13	81.8 ± 3.08
		5	70 ± 0.02	4 ± 1.62	55.3 ± 0.64

Table S7. Summary of the encapsulation efficiency, loading capacity (target and actual), and burst release of high BSA loading formulation (8% and 15 %) w-o-w and sg-o-w microparticles

Protein	Theoretical protein loading (%)	Emulsion method	Encapsulation Efficiency (%)	Loading Capacity (%)	24th hour release (%)
BSA	15%	w-o-w	17 ± 1.63	2.6 ± 0.23	95
		sg-o-w (95% SGnP [1:20] loading)	40 ± 4.41	6 ± 0.72	91
	8%	w-o-w	31 ± 4.08	2.5 ± 0.32	80
		sg-o-w (48% SGnP [1:20] loading)	54 ± 5.12	4.5 ± 1.51	26

From Tables S8 (i, ii, iii and iv), we have determined the ratio of active to inactive GF (determined by ELISA and consecutive total GF released (active plus inactive parts determined by NanoOrange) on each time point of the release study. The last columns in Table S8i, 8ii, 8iii and 8iv indicates the average ratio of active to inactive EGF released from w-o-w samples, the average active to inactive ratio of FGF-2 released from w-o-w samples, the average ratio of active to inactive EGF released from sg-o-w samples and the average ratio of active to inactive FGF-2 released from sg-o-w samples, respectively.

For the dual GF (EGF and FGF-2) release study from w-o-w and sg-o-w samples, we can only ascertain the amount of active and total protein. We could not measure the inactive fraction of EGF/FGF-2 in the release medium. However, in Tables S8v and 8vi we have roughly estimated the amount of inactive GF released from each formulation by using the ratio of active to inactive GF determined from the previous tables (i, ii, iii, and iv for release study of individual EGF or FGF-2). Although this is a rough estimation, yet we can see that the amount of release of estimated total individual GF from the dual GF loaded systems is closely tally that of the actual total GF release. The little over estimation is obvious as the average ratio (active to inactive) we considered in the estimation, was observed to vary in different release samples as you can see Table S8i to 8iv. However, the estimation provides a qualitative assessment to validate our results.

Table S8. Summary of the release profile of FGF-2 and EGF as single and dual protein loading via w-o-w and sg-o-w emulsion. The active fraction of the GF is quantified by respective ELISA and the total protein (active and inactive fractions) is quantified by NanoOrange (NO).

(8i)	EGF release from w-o-w microparticles						
Release day	Amount of EGF eluted estimated by ELISA (ng)	Percentage of active EGF fraction estimated by ELISA with 243 ng as starting amount (%)	Total EGF (active+inactive) released determined by NanoOrange (ng)	Percent of total EGF (active and inactive) (%)	Amount of inactive EGF estimated (ng)	Ratio of active to inactive EGF	Average ratio of active to inactive EGF ± SD
1	18.1	7.45	79.09	32.55	60.99	0.3	0.47 ± 0.19
2	32.81	13.5	155.92	64.16	123.11	0.27	
3	54.82	22.56	194.15	79.9	139.33	0.4	
5	70.47	29	204.86	84.3	134.39	0.52	
7	78.98	32.5	212.41	87.41	133.43	0.6	
10	97.2	40	224.92	92.56	127.72	0.76	

(8ii)	FGF-2 release from w-o-w microparticles						
Release day	Amount of FGF-2 eluted estimated by ELISA (ng)	Percentage of active FGF-2 fraction estimated by ELISA with 407 ng as starting amount (%)	Total FGF-2 (active+inactive) released determined by NanoOrange (ng)	Percent of total FGF-2 (active and inactive) (%)	Amount of inactive FGF-2 estimated (ng)	Ratio of active to inactive FGF-2	Average ratio of active to inactive FGF-2 ± SD
1	46.81	11.5	134.31	33	87.5	0.13	0.4 ± 0.5
2	80.59	19.8	175.01	43	94.42	0.21	
3	130.65	32.1	219.78	54	89.13	0.36	
5	144.89	35.6	256.41	63	111.52	0.32	
7	158.73	39	313.39	77	154.66	0.25	
10	223.85	55	329.67	81	105.82	0.52	

(8iii)	EGF release from sg-o-w microparticles						
Release day	Amount of EGF eluted estimated by ELISA (ng)	Percentage of active EGF fraction estimated by ELISA with 320 ng as starting amount (%)	Total EGF (active+inactive) released determined by NanoOrange (ng)	Percent of total EGF (active and inactive) (%)	Amount of inactive EGF estimated (ng)	Ratio of active to inactive EGF	Average ratio of active to inactive EGF ± SD
1	25.6	8	32	10	6.4	1.25	3.5 ± 1.6
2	33.28	10.4	41.6	13	2.6	4	
3	46.4	14.5	54.4	17	2.5	5.8	
5	54.08	16.9	70.4	22	5.1	3.31	
7	56	17.5	73.6	23	5.5	3.18	
10	67.2	21	144	45	24	0.88	

(8iv)	FGF-2 release from sg-o-w microparticles						
Release day	Amount of FGF-2 eluted estimated by ELISA (ng)	Percentage of active FGF-2 fraction estimated by ELISA with 378 ng as starting amount (%)	Total FGF-2 (active+inactive) released determined by NanoOrange (ng)	Percent of total FGF-2 (active and inactive) (%)	Amount of inactive FGF-2 estimated (ng)	Ratio of active to inactive FGF-2	Average ratio of active to inactive FGF-2 ± SD
1	18.9	5	30.24	8	11.34	1.67	11.5 ± 7.5
2	34.02	9	37.8	10	3.78	9	
3	64.26	17	71.82	19	7.56	8.5	
5	68.04	18	75.6	20	7.56	9	
7	75.6	20	79.38	21	3.78	20	
10	79.38	21	83.16	22	3.78	21	

(8v) Dual GF (EGF and FGF-2) loaded w-o-w microparticles

Release day	Amount of EGF eluted estimated by ELISA (ng)	Percentage of active EGF fraction estimated by ELISA with 165 ng as starting amount (%)	Amount of inactive EGF released estimated from ratio from SI Table 8 i (ng)	Total EGF (active+inactive) estimated (ng)	Estimated percentage of total EGF(active+inactive) release (%)		
1	30.86	18.69	65.66	96.52	58.5		
2	33.17	20.11	70.57	103.74	62.87		
3	56.11	34.02	119.38	175.49	106.36	Total protein (EGF+FGF 2) released estimated by NanoOrange (ng)	Percent of total protein (EGF+FGF-2) released estimated by NanoOrange (%)
5	59.42	36.01	126.43	185.85	112.64		
7	61.05	37	129.89	190.94	115.72	150.36	48.04
10	64.35	39	136.91	201.26	121.98	196.36	62.73
Release day	Amount of FGF-2 eluted estimated by ELISA (ng)	Percentage of active FGF-2 fraction estimated by ELISA with 148 ng as starting amount (%)	Amount of inactive FGF-2 released estimated from ratio from SI Table 8 ii (ng)	Total FGF-2 (active+inactive) estimated (ng)	Estimated percentage of total FGF-2 (active+inactive) release	230.15	73.53
						248.56	79.41
1	25.16	17	62.9	88.06	53.37	288.45	92.16
2	29.01	19.6	72.53	101.54	61.54	297.66	95.09
3	48.25	32.6	120.63	168.88	102.35		
5	58.91	39.8	147.28	206.19	124.96		
7	66.01	44.6	165.03	231.04	140.02		
10	74.44	50.3	186.1	260.54	157.9		

(8vi) Dual GF (EGF and FGF-2) loaded sg-o-w microparticles

Release day	Amount of EGF eluted estimated by ELISA (ng)	Percentage of active EGF fraction estimated by ELISA with 185 ng as starting amount (%)	Amount of inactive EGF released estimated from ratio from SI Table 8 iii (ng)	Total EGF (active+inactive) estimated (ng)	Estimated percentage of total EGF(active+inactive) release (%)		
1	7.4	4	2.11	9.51	5.14		
2	12.95	7	3.7	16.65	9		
3	14.8	8	4.23	19.03	10.29	Total protein (EGF+FGF 2) released estimated by NanoOrange (ng)	Percent of total protein (EGF+FGF-2) released estimated by NanoOrange (%)
5	15.73	8.5	4.49	20.22	10.93		
7	16.65	9	4.76	21.41	11.57	21.63	6.11
10	18.5	10	5.29	23.79	12.86	41.3	11.67
Release day	Amount of FGF-2 eluted estimated by ELISA (ng)	Percentage of active FGF-2 fraction estimated by ELISA with 169 ng as starting amount (%)	Amount of inactive FGF-2 released estimated from ratio from SI Table 8 iv (ng)	Total FGF-2 (active+inactive) estimated (ng)	Estimated percentage of total FGF-2 (active+inactive) release	51.13	14.44
						53.1	15
1	10.14	6	0.88	11.02	6.52	55.07	15.56
2	11.83	7	1.03	12.86	7.61	59	16.67
3	20.28	12	1.76	22.04	13.04		
5	23.66	14	2.06	25.72	15.22		
7	28.73	17	2.5	31.23	18.48		
10	35.49	21	3.08	38.57	22.82		